

Thirty years of DNA — and after

Molecular biology, and the genetic material called DNA that it made fashionable, is only thirty years old. Most people will be tempted to believe that time has slipped. But that is the sense that true discoveries create.

In the conventional or at least widely taught description of the process of discovery, discovery as such is almost unimportant. According to Thomas S. Kuhn (now at MIT), the times now recognized as turning points in the development of science are marked not by discoveries but by the recognition that it is no longer possible to preserve inviolate old ways of thought, to save the appearances as Newton said (in Latin), and that the time has come to seek comfort in some other pattern. Thus, Kuhn might say, geophysicists held on to the doctrine that a rigid Earth whose surface features are immovable has repeatedly been remodelled by physiogeographical processes until the effort ceased to be worthwhile, whereupon they did what they might have done half a century earlier — resurrected Wegener and then invented the name plate tectonics to stand for what Wegener called continental drift. The trouble with Kuhn's view of the phenomenon of discovery is not merely that it is at once too cynical and too Olympian, but that it is wrong.

If Kuhn should ask for an illustration of the error of his belief that the great turning points are better described as paradigm-shifts than as discoveries, he should give some thought to what happened thirty years ago, when J.D. Watson and F.H.C. Crick sent off a commendably brief "Letter to *Nature*" describing "A structure for deoxyribonucleic acid". For at that time, there was a nagging question to be decided — what is the physical vehicle of inheritance, protein or something else? By then, to be sure, the view that proteins might be the materials of which genes, classically defined, are made had been abandoned by people in the know. The notion that that role might have to be ascribed to chemically featureless nucleic acids was for most people an acknowledgement of bankruptcy.

The circumstances of thirty years ago are thus exactly comparable with those preceding Newton's formulation of his theory of the Moon. By then, it had become plain that the old Aristotelian explanation of celestial events would not suffice, it was clear that some other explanation must supervene but nobody could guess what that might be. The intellectual world was, for a time, suspended between doubt and certainty. That Newton should have exorcised this quandary by such a simple remedy as the inverse-square law was, to many of his contemporaries (Descartes among them) an offensive piece of good luck. So it was (and is) with Watson and Crick. "A structure for deoxyribonucleic acid" is merely a latter-day equivalent of Newton's inverse-square law. The fact that the discovery is presented in words, not formulae, and with a diagram drawn out of scale, is merely a reflection of the difference of the subject. For the overwhelming truth is that the reasons why Watson's and Crick's short paper should have swept the world is that, like Newton's *Principiae*, it answered a question that people had been asking. And it did so so neatly that nobody, hardly anybody, could dissent. We are fortunate (but a growing number of us are too young) to have witnessed such a cogent case for the transformation of an intellectual paradigm (as Kuhn would say) derived from such a simple discovery. This remark is not intended to assert that the authors of that thirty-year-old paper should be given sinecures as joint directors of the British Royal Mint (for which each would probably wish to be disqualified) but merely to acknowledge the importance of what they did.

By good fortune, *Nature* has been an interested bystander in

these events, which is why it is organizing a conference on "Thirty Years of DNA" in Cambridge this week and in Boston next September. Watson and Crick sent their paper to this journal no doubt partly out of habit, partly for the sake of rapid publication and partly because they thought that what they had to say deserved a generous readership. *Nature*, merely the medium and not the message, has vicariously been one of the beneficiaries. The genetic code is indeed a triplet code, as the simplest arithmetic suggests it should be. There is an enzyme best-called reverse transcriptase (found independently by Baltimore and Temin) that will turn RNA into a DNA copy of itself which is nevertheless not an offence against Crick's "central dogma" that "DNA makes RNA makes protein" but rather the exception that proves the rule. It is literally marvellous that such a simple idea should have swept so much before it.

But why not hold a celebratory conference at some more natural time, twenty-one years after the event or even twenty-five years after? Because at that stage the promise of what Watson and Crick offered thirty years ago had not been fulfilled. The jibe then often offered by clinicians that molecular biology had done nothing for patients was still sustainable: people might have a better understanding of disease, but they were no better able to cure it. Now, five years later, remarkable changes have occurred. The report two weeks ago suggesting how a vaccine against malaria might (or, rather, will) be constructed is only as straw in the wind. This week's account of how adult T-cell leukaemia may be carried by a virus that infects people would, likewise, not have been possible without the techniques now available for telling one stretch of DNA from another. And what other route can there be to the understanding of acquired immune deficiency disease, to which homosexuals appear predominantly at risk? Looking at the DNA of those affected is the only start. Telling how that DNA differs from the normal is the immediate objective. But hoping to tell how it became like that has become possible only in the past five years. And so it is with many other genetic diseases, malignancies among them.

The application of these new techniques in other fields, agriculture or even the chemical industry, has been much canvassed in recent years. And it is entirely possible that, in the course of time, new-style botanists will find a way of grafting nitrogen-fixing genes onto ordinary plants, or that chemical engineers will find means for catalysing reactions not ordinarily possible by using chemical not yet made. For the next ten years or so, however, what matters most is that the understanding of molecular biology we have acquired will throw light chiefly on the mechanism of several forms of disease and thus point to ways in which those diseases can or may be cured. A mere decade from now it will be no more possible for a person to pretend that he is indifferent to the logical relationship between the structure of the DNA that his cells contain and his external characteristics — his phenotype — so that even questions of whether intelligence (whatever that may be) is inherited or heritable will become respectable for the first time since the nineteenth century.

By all accounts, Watson and Crick thirty years ago had no intention of raising such subversive questions. Like the Englishmen to whose university they once both belonged, they have done their best over the past thirty years to keep a straight bat, to be impartial. Most probably Watson and Crick have in the



process given comfort to those among their critics who suppose it to be an offence that people should believe that the world and even living things can be understood. For the truth is that by helping to throw light on the reasons why living things can be in some respects better understood as machines, they have unwittingly advanced the cause of rationalists, reductionists or whatever. So what, is Watson's and Crick's defence? Our purpose was simply to tell it like it is, and to train a few talented students in the process. If the Kuhns of this world seek to find a paradigm-shift, surely that is their business and not ours? How else would Newton have spoken?

The Watson and Crick discovery thirty years ago has worked two other magics. We may all be a little more reductionist than we used to be, but we are also collectively more skilled and more confident. Skill is too often demeaned, as if it were merely a craft. But suppose that the practice of the skill lies in the telling of the difference between a regular gene and an aberrant version of it, differing by one nucleotide in a thousand, and which in its altered form resembles the gene of a known retrovirus? Is that demeaning work? Or a way of helping to understand malignancy and get rid of it?

We are also more confident. In the south of England the celebrated tourist attraction called Stonehenge is also now recognized to be one of the first scientific observatories, given over to the provision of a calendar for a supposedly primitive people, only just converted from pastoral to agrarian ways. The modern marvel is that such primitive people should have assembled such a massive structure, incorporating massive stones carried from scores of miles away. The contemporary marvel must have been that the construction of a calendar should have been worthwhile. Taxation in aid of the enterprise would have weighed heavily on those concerned — they would have had to be forced to help move all those stones. Most of the labourers would have been dead by the time Stonehenge was completed. Their successors, children or more probably grandchildren, would however have benefited from knowing when best to plant their corn. They would have done so more confidently than previously, and would have been less afraid of other irrational assaults by nature. We are lucky that our modern equivalent of that bronze-age monument has been constructed so much more quickly, with so much less effort and with so much excitement for us all.

Who should test drugs?

The latest case of scientific fraud in the United States presents FDA with a challenge

CASES of fraud in research seem almost to follow a familiar script: a bright young researcher, working in a large laboratory with little supervision but under pressure to be "productive", is tempted first to cut corners and later to manufacture results wholesale. The outcome also follows the script. Sage words are uttered by senior people either about the triumph of the self-correcting process of science (because the culprit was discovered) or about the bankruptcy of the peer review system (because discovery took so long). The case of Dr Wilbert S. Aronow (see p. xxx) is a departure from this script. Dr Aronow, until last summer chief of the cardiovascular section at a Veterans Administration Hospital in California, was hardly a young man in need of proving his ability to perform. A well respected cardiologist, author of innumerable scientific papers, consultant to 21 scientific journals, principal investigator to the Food and Drug Administration (FDA) itself — the government agency that discovered his falsification of data — Aronow had nothing to gain and everything to lose. And in his case, more was at stake than the reputation of the research enterprise — potentially, the health and well-being of thousands of heart patients.

FDA, charged by law with reviewing drug companies' claims for the safety and efficacy of their products, is acutely aware that it cannot rely on the usual check provided by peer review. As well

as a conventional evaluation of the results and methodology of clinical trials submitted by the drug companies, the agency conducts more than 200 audits each year. Investigators are despatched, for the most part on a routine and random basis, to pore over the records of clinical trials and to make sure that results reported match results obtained. It was through such an audit that discrepancies in Aronow's reports were uncovered. It can be argued that justice was properly served by FDA's conclusion of the case. Aronow signed a pledge that he would not participate in further clinical trials, sponsors of his research have been informed and he himself has paid a high personal price, of which loss of his post will seem only a small part.

But the case raises some deeply troubling questions. What can FDA do — and what is it willing to do — about deliberate falsification of the data on which it relies to judge the safety and effectiveness of new drugs? Unusual as Aronow's case may be, it raises the question of how many other researchers have at least felt the pressures (which apparently overcame Aronow) to produce results that make their drug-company sponsors happy. FDA's investigator noted that all of Aronow's discrepancies favoured the drugs' efficacy. And surely even the most incorruptible researcher is not immune from wondering where his next research dollar is to come from.

FDA has long maintained that it is easier to scrutinize the research of others than to do the work itself — not only easier but more likely to yield the truth. There is much in that contention, and it is easy to imagine the litigative nightmare that could be created if FDA performed all tests itself. Each unfavourable finding could be challenged by the drug companies, which meanwhile would have commissioned a private study anyway. The regulation of automobile safety and exhaust emissions (by the Environmental Protection Agency) is already a fine model of that nightmare. But FDA and the drug companies also maintain that it is in the companies' own best interest to ensure that the studies are accurate. Certainly this is true in the long run, particularly with regard to safety. But this argument is suspect when it comes to claims of efficacy. The battles FDA is continually waging over what the companies are permitted to claim for their products in advertising reveals the consuming need that possesses the companies to press efficacy claims to the limit, and not infrequently beyond. Aronow's case comes to light at a time when FDA is in the process of relaxing many of its requirements on the submission of data for new drug approval. Individual case report forms will no longer be required on a routine basis and foreign studies, which have often proved difficult to audit, will be accepted more readily. In so doing, FDA may be sending out the wrong signal at the wrong time. There is ample evidence that tighter scrutiny is what is called for.

FDA may also need to reconsider its procedures for disqualifying researchers who violate its regulations. The process has grown more cumbersome every year since its institution, to the point where it can now take more than three years from the time evidence of wrongdoing is discovered to the time the researcher is officially barred from conducting clinical trials. The proceedings have begun to take on the trappings of a court of law, complete with lawyers, delays and obfuscation. FDA officials concede that it is because of the cumbersomeness of the procedure that the agency has been willing to settle for consent agreements as in Aronow's case — by which the researcher pledges not to participate in clinical trials and by which he is spared the embarrassment of finding his name appended to a public list of disqualified researchers. But this solution does not fully serve the public interest. Journals that had published Aronow's questionable results, and the Environmental Protection Agency which had relied on Aronow for key studies of the health effects of carbon monoxide, had to learn of FDA's findings through an account leaked in the *Washington Post* months after the consent agreement was signed. FDA should not feel obliged to burden itself with quasi-legal proceedings in order to disqualify unreliable researchers. Conducting a clinical trial on a new drug is hardly a right protected by guarantees of due process — it is a privilege that carries with it the profound trust of society.

Medicine in France

Students, interns in protest about examinations

Do not arrange to be sick in France just now. Medical students, interns and heads of clinics in teaching hospitals are all on strike. Professors are taking over the more menial duties of patient care, and patients are turning to private medicine. (The massive French social security system pays back up to 80 per cent of the fees.) The overt issues are new examinations (for the student) and security of employment (for the interns and clinic chiefs). But a general malaise of the medical system and uncertainty about the future may be the true cause.

The students have been on strike since 22 March, and last week their action boiled over into demonstrations in many major towns: roads were blocked and signs were daubed in Nice, barricades were burned in Marseilles, paving stones torn up in Bordeaux and the riot police were out in Paris.

The scenes evoked memories of 1968, when all the universities took part in demonstrations on the streets. And 1968 plays a part in the present troubles. After the "revolution", examinations were softened, spread out into frequent assessments along a six-year course with no final examination. Now the Socialist government, in a reform whose origins date back to the previous administration of Giscard d'Estaing, wants to re-establish a system of final examinations covering all the work of the course.

In fact the government wants two examinations, the first general and the second, to be taken three months later, covering a specialist field of the student's choice. But the real crunch is that the first examinations would be set next year, so a large number of students now well into their studies based on continuous assessment would be forced to face a further, and unexpected, hurdle. Hence the riots. The government, meanwhile, stands firm and offers no compromise — although professors quietly expect that there will be one that takes the form of delay, so that the examinations will not affect the existing student body, but will be phased in with all future intakes. The general feeling seems to be that it is reasonable to have an examination but unreasonable to impose it quite so suddenly.

As for the interns and *chefs de clinique*, their problems are different. They are complaining about their salaries and job insecurity. To become an intern at a teaching hospital (of which there are 13 in Paris, and often two in other major cities) is about as difficult as joining one of the

grandes écoles and is similarly considered to be a privilege. The job, like its equivalents in other countries, is a four-year mixture of training and patient care. The pay is appropriate to a student, but too little for the responsibilities of the job and the age of the incumbents, say the interns.

Moreover, as elsewhere, internship and the following post of *chef de clinique* are not careers: at the end of four years, an intern can move on to be *chef de clinique*, again a four-year post, after which the student must pass an examination to become an assistant professor (a permanent post) at the teaching hospital, or move out into private practice. But private medicine has been contracting in France for some years, and the interns want new guarantees of employment: they demand either that internship (or the *chef de clinique*) be considered permanent posts, or that the number of assistant professorships awarded each year be increased (it was 85 last year, and the interns seek 500) or that they have an

exclusive route out into the public non-teaching hospital parallels of assistant professorship. At present, those jobs are filled in part from teaching hospital interns and in part from elsewhere: the teaching hospital interns demand exclusive and contractual rights to be considered first for these jobs.

Again, as with the students, the government appears to be inflexible. The previous, Communist minister of health, Jack Ralite, fell in the recent reshuffle to be replaced by Socialist Edmund Hervé (ex-junior minister of energy) and everyone is waiting to see where his sympathies lie; but meanwhile, the government appears to have shifted the problem onto the minister of social services, Pierre Bérégovoy, who has a widespread reputation as a trouble-shooter.

He may succeed in this instance — but at root the French troubles may lie deeper than even Bérégovoy can reach. Despite the shrinking private sector, a working public health system is still not properly established in France, many in the present government feel.

Meanwhile, as with all national health systems, costs burgeon and government thoughts, particularly a Socialist government's thoughts, turn to radical reform. In the medical community, therefore, insecurity reigns, and the strikes are a symptom of it.

Robert Walgate

French research

Fabius promises no change

RESEARCH is safe with Laurent Fabius! That, at least, is how the new minister of industry and research in France declared himself last Monday. On his own recognisance, M. Fabius fully supports the ambitious initiatives of his predecessor, Jean-Pierre Chevènement.

Making his first major speech in his new post (he was previously the budget minister, holding Chevènement's purse-strings among others), Fabius declared that he "supported every promise" of the law for science passed by the French Parliament last year. He supported the "*programmes mobilisateurs*", (the seven areas of special support singled out by Chevènement), the principle of opening science to industry and generally breaking down barriers to communication and cooperation and the goal of raising total French civil research and development spending to 2.5 per cent of the gross national product by 1985. Despite the cost, this was "an indispensable effort", he said.

Earlier, Fabius had announced through the French press agency (AFP) that he now felt himself "a spokesman for the future" — obviously relishing his new position — "I know that the scientific community is now mobilized and prepared to make research a national priority. I feel today — and it is a great responsibility — the inheritor of this trust."

Thus in many quarters of French science there must be a feeling now that M. Fabius may not prove to be the scourge that had been feared. Nevertheless, the details of the revised French budget for 1983, taking into account new austerity measures, have yet to be announced, and pessimists point out that Fabius has committed himself only to 2.5 per cent by 1985.

Earlier this year, before the reshuffle, the Centre National de la Recherche Scientifique (which supports most basic research) announced a supposedly temporary spending freeze, limiting spending to 60 per cent of previously agreed figures; and the medical research council INSERM more optimistically restricted itself to 80 per cent. These restrictions still stand, and if confirmed would certainly eliminate the increases achieved under the previous minister. Thus a meeting of the council of INSERM this week to discuss budgets is described by one director of a major laboratory as "of prime importance for my laboratory's survival in 1984".

French scientists are now waiting impatiently to see what the rhetoric of M. Fabius looks like when it comes to hard francs. M. Chevènement's example is awesome, both for its new minister and for the government of which he was a part.

Isabelle Trocheris

Data falsification

Food and drug data fudged

Washington

A PROMINENT cardiologist, Dr Wilbert S. Aronow, has agreed to disqualify himself from future new drug trials in the face of evidence that he submitted false data to the Food and Drug Administration (FDA) on the efficacy of several drugs being tested for FDA approval. FDA has ordered drug companies to produce supporting evidence for the safety and efficacy of all drugs previously tested by Aronow.

FDA's findings have also provoked an investigation by the Environmental Protection Agency (EPA) of several studies by Aronow of the effect of low levels of carbon monoxide (CO) on heart patients, which figure prominently in EPA's ambient air standard for CO, which is in the process of being revised.

Aronow, formerly chief of the cardiovascular section at the Veterans Administration (VA) Hospital in Long Beach, California, has conducted a "vast number" of new drug investigations, according to FDA. For several years he was a member of FDA's advisory committee on cardiology. According to FDA documents, Aronow apparently signed the agreement not to participate in future drug studies in order to avoid formal "disqualification" proceedings. Aronow resigned from the VA Hospital last summer and is now at Creighton University Medical School in Omaha, Nebraska.

His troubles began in the summer of 1979, when FDA officials were going over, as a matter of routine, a study he had performed on the drug Minipres (prazosin). His study had been sponsored by Pfizer, the drug's manufacturer, which was seeking FDA approval to market it as a treatment for congestive heart failure. The drug was already on the market as an antihypertensive. Aronow's results seemed too perfect in showing the drug's success in treating congestive heart failure, and FDA investigators made an appointment with Aronow to go over his data.

The day before the investigators were to arrive, Aronow, apparently in a state of panic, called Dr Marion Finkel, associate director for new drug evaluation, and, admitted that he had "fudged" X-ray reports from his studies of prazosin and one other drug. A note by Finkel of the conversation says "The reports he submitted to the sponsors were different from the radiologists' interpretation of those X-rays. He was very upset about the matter, explained that he had been under an emotional strain, that that accounted for the alteration of the X-ray findings and he would never do anything like it again."

The audit was conducted as planned, and turned up further discrepancies. The FDA investigator reported that only one out of 10 X-rays examined matched Aronow's case reports. And in an affidavit

that Aronow then signed, he admitted that he had not even examined most of the X-rays himself, but had simply filled in the case reports to match his beliefs about the drugs' effectiveness.

Serious problems were also found in Aronow's study of the drug timolol which was supposed to test the drug's effectiveness in reducing angina attacks. The subjects were supposed to be patients who were experiencing frequent angina attacks, but five of them had in fact experienced none or at most one per week before the test began. Aronow reported that the subjects' pre-test rate was as high as 14 per week — a discrepancy that would exaggerate the apparent effectiveness of the drug.

Other records could not be found. Aronow admitted discarding them, even though he was aware of the legal requirement to retain all records for two years.



In all, FDA found irregularities in five studies involving four different drugs between 1974 and 1978. The FDA investigator reported that "all discrepancies noted were on the side of favoring drug efficacy".

Aronow later retracted his admissions. At a meeting with FDA officials in 1980, he excused the X-ray discrepancies by saying that "the radiology residents' reports are considered to be very unreliable", and that he had therefore gone over all the X-rays himself and arrived at a different interpretation. And he said that when he confessed to Finkel and when he signed the affidavit, he was in state of severe emotional distress. Aronow made repeated reference to the fact that he has been under a psychoanalyst's care for the past several years. But while denying FDA's charges, Aronow again promised not to participate in further drug studies without FDA's approval.

FDA documents show that the agency's officials were clearly dissatisfied but that they decided to accept the promise and drop formal disqualification proceedings.

The final consent agreement, reached in October 1982 (but revealed only two weeks ago in the *Washington Post*), confirms Aronow's pledge, and provides that the only outside parties to be informed of his voluntary disqualification were sponsors of drug studies he had conducted. He was thus spared having his name published on a list of "Investigators ineligible to receive investigational new drugs", which is one consequence of official disqualification.

According to Dr Alan Lisook, chief of FDA's clinical investigations branch, the agency has reached similar agreements in seven other cases. Lisook said the consent agreement provides a "short cut" to the cumbersome official disqualification procedure, which can take more than three years. Fifty researchers have been officially disqualified nonetheless.

Aronow's involvement in key studies for EPA has raised some questions about the appropriateness of an unpublicized consent agreement. EPA officials learned of FDA's investigation only through the *Washington Post* article. EPA has quickly assembled a committee of four "well-recognized experts" to meet Aronow and review the studies he did for the agency.

The studies played a pivotal role in the development of the agency's proposed ambient air standard for CO. By law, these standards must be stringent enough to protect the most sensitive population — and according to Aronow's studies, the most sensitive population consists of angina patients, who, he found, experience earlier onset of symptoms at levels as low as 2 per cent carboxyhaemoglobin (COHb).

Kent Berry of EPA's office of air quality planning and standards said the agency had received some comments on its proposed standard that "flag the problem that the data [at 2 per cent COHb] looked too consistent."

But EPA officials insist that there is no reason as yet to doubt Aronow's results and that it is "inappropriate for people to be jumping to conclusions about these studies". The committee reviewing the data is to report by the end of the month, and no final action on the proposed CO standard will be taken before then.

Another question raised by the unpublicized agreement is whether FDA should have informed journals that published the questionable research. FDA's Lisook said that although Aronow's prazosin study was published in *Circulation*, it was decided not to demand a retraction because other independent data supported the overall conclusion. Lisook said that FDA has demanded retractions in other cases.

But the editor-in-chief of *Circulation*, Dr Elliot Rapaport, took a different view. Like EPA, he learned of the questions about Aronow's work from newspaper reports. "I would have to say it was inappropriate for them [FDA] not to notify us if that data was faulted", he said.

Stephen Budiansky

Environmental lead

British studies stress social factors

THE Department of the Environment announced last week that investigations by three major research groups have failed to detect statistically significant effects of lead on behaviour and intelligence of children in three British cities. The studies, which have not yet been published in full, are claimed to support the idea that any differences in behaviour between lead level groups are due to social factors that have not before been investigated in detail. The timing of the announcement and the interpretation placed on the results have been criticized by the Campaign for Lead-Free Air (CLEAR).

The studies were carried out at the Institute of Psychiatry, the Institute of Child Health and the University of Birmingham. All subjects had lead levels that are normal in Western societies and which fall within EEC reference levels. Two of the research groups originally found significant IQ deficits of 5 to 7 points in high lead groups, but these were reduced and became statistically insignificant when social factors were taken into account and sample sizes increased. The investigators see no inconsistency with previous US and West German research that suggests a negative correlation between IQ and lead levels in children from low income families: social factors were analysed in more detail in the new studies and American children appear to have higher lead levels than their British counterparts.

A spokesman for CLEAR accused the government of trying to defuse public reaction by issuing its statement before full details of the studies are available. CLEAR regards at least one of the new studies as supporting its case for a complete ban on lead in petrol (gasoline). The Department of the Environment says its statement was issued last week simply because the studies themselves were to be made public at a meeting of the British Psychological Society on 10 April.

Clear says the consistent residual differences in behaviour between groups with different levels of lead in their blood that have been found in most recent studies, especially in low-income groups, point to an overall association between lead levels in the body and IQ deficit. Professor Derek Bryce-Smith, a pioneer of the anti-lead campaign, has said that social effects cannot simply be removed from the analysis because the toxic effects of lead are known to interact with social variables. Ms Marjorie Smith of the Institute of Child Health, who worked on one of the studies, says this objection has been avoided in her analysis and that residual differences between groups are to be expected if all studies are "making the same mistakes".

The latest results will be influential in the debate over whether lead additives in petrol should be banned completely. The Royal

Commission on Environmental Pollution is due to report next week on lead pollution, and it is thought that the commission has been able to examine data from at least one of the latest studies. The British Government is already committed to reducing lead levels in petrol by the end of 1985, from the present maximum of 0.4 grammes per litre to 0.15 grammes per litre. A recent nationwide survey of population lead levels carried out under the European Community's screening programme found levels in Britain to be generally within reference levels, with the exceptions attributable to localized sources. But support is growing in the European Parliament for a total ban on lead in petrol sold in member states. Studies attempting to assess the proportion of lead in the body that is due to

petrol additives have produced estimates ranging from 10 per cent to more than 50 per cent. CLEAR says that blood level reductions in the United States and in Japan following statutory petrol lead reductions point to the higher figure.

If there is, as seems likely, pressure on the Government to reduce lead emission further, filters fitted in car exhaust systems could provide a practicable alternative to reducing lead content in fuel. Filters developed by Associated Octel, which manufactures lead additives for petrol, are claimed to reduce lead emissions by 70 to 80 per cent over a 50,000 miles working life. Figures produced by the Department of Transport show that a policy of fitting the filters, which contain steel wool coated with a layer of alumina, could be implemented about five years sooner than a policy requiring all new cars to run on lead-free petrol — and at lower cost.

Tim Beardsley

Arms control**Last week's developments**

As a service to readers interested in arms control, and in the hope of not boring others with over-long articles on the subject, Nature will in the months ahead publish occasional summaries of the previous week's developments. The first follows.

THE log-jam in the working group on a treaty on chemical weapons has been broken by Soviet acceptance of the Canadian chairman, Mr Don McPhail. (Other working groups set up by the Geneva-based Committee on Disarmament remain hamstrung by disputes about chairmanships.) The British proposals on verification put forward last month are said to have been well received, but a response from the Soviet side is not expected for some weeks.

The report of the US bipartisan commission on MX siting, which has been widely leaked, is said to propose as an interim measure putting 100 MX missiles in as many strengthened Minuteman silos and to advocate the development of a smaller (and thus potentially mobile) single-warhead missile, called the Armadillo, for the longer term. The question whether Congress will agree to pay for the interim solution has been raised in Washington, together with the suggestion that a single-warhead missile would require that a strategic weapons treaty should be based on a count of warheads, not launchers.

The basis of the US Administration's proposals to the Strategic Arms Reduction Talks (Start) at Geneva, given to a congressional committee in Washington before Easter by the chief negotiator, Edward L. Rowny, have now become public. The United States is advocating a force of 850 strategic missiles on each side — the equivalent of a two-thirds reduction by the Soviet Union and a reduction of nearly a half by the United States. Nothing is known of the Soviet response.

Meanwhile, the US Administration's

decision not to insist on the "zero option" for the proposed treaty on intermediate-range nuclear missiles, widely welcomed by Western governments, has been rejected (at least in public) by the Soviet Union — most authoritatively by Mr Andrei Gromyko in Moscow on 3 April. The US proposal is that the Soviet force of SS20 missiles should be reduced to some number (unspecified) that would also be an upper limit to the number of Pershing II and cruise missiles to be deployed in Western Europe in the next three years. Mr Gromyko said during a televised press conference that the proposal was unacceptable because it would constrain Soviet deployment of SS20 missiles not aimed at Western Europe and because it left out of account the British and French nuclear missiles. Nothing was said about aircraft (in which the Soviet Union is thought to have a numerical advantage) nor about battlefield nuclear weapons (where NATO forces are thought to be better-equipped).

Reports persist that the US Administration considers a Soviet missile test on 8 February to be a violation of the Salt II agreement, which forbids the development of more than one further land-based strategic missile system, and that the issue will be raised in a speech by President Reagan in a few weeks. By then, Mr Reagan will know whether the Senate will let him have Dr Kenneth Adelman as director of the Arms Control and Disarmament Agency and whether the House of Representatives will have passed a resolution supporting a nuclear freeze proposal. □

Technological trade

Congress cool on new restrictions

Washington

PRESIDENT Reagan asked Congress last week for stronger powers to staunch what the Administration has described as a haemorrhage of American scientific and technological secrets to the Soviet Union. But the proposals, contained in new legislation drafted by the Department of Commerce to replace the expiring Export Administration Act, were given an unsympathetic reception on Capitol Hill.

The present act authorizes the government to control the flow of exports for political or national security reasons and was used last summer to enforce the president's embargo on machinery destined for the Soviet-European natural gas pipeline. It has also been used to prevent scientists from publishing or "exporting" sensitive research, most notoriously in 1980 when commerce officials made the American Vacuum Society exclude Soviet and East European experts invited to a symposium on magnetic bubble devices.

Exporters had hoped that expiration of the act next September would be used as an opportunity to relax some controls and

restore the sanctity of international contracts. Instead, Mr Lionel Olmer, under secretary for international trade administration, told a House of Representatives foreign affairs subcommittee that the administration would not allow the needs of exporters to overshadow the "high priority" of keeping sensitive Western technology out of the hands of the Soviet Union.

The new bill would change the language of the act to emphasize the overriding importance of national security, and proposes new statutory crimes for attempting to violate the export regulations. But the most significant change is a new emphasis on efforts to stop third countries passing Western technology to the Soviet Union.

One measure proposed in the bill, to strengthen the operations of COCOM, the Coordinating Committee of NATO nations (minus Iceland and plus Japan) responsible for regulating the transfer of technology to the Eastern bloc, has wide support in Congress. There is, however, strong opposition to a provision enabling the President to stop foreign companies

believed to have violated the act's national security regulations from importing their goods to the United States.

It was precisely this measure that caused disarray in the Western alliance last summer during the pipeline controversy, and its appearance in the draft legislation is expected to attract international criticism at the forthcoming economic summit at Williamsburg, Virginia. Representative Donald Bonker, the Washington state Republican who chairs the foreign affairs economic policy and trade panel, warned Mr Olmer last week that a zealous pursuit of foreign trade sanctions was likely to weaken rather than strengthen cooperation between the COCOM nations.

The bill does contain some concessions designed to pacify exporters. Existing contracts could be honoured within 270 days of the imposition of any new trade sanction imposed by the president. The Department of Defense has been asked to decontrol the export of scientific equipment containing microprocessors, which at present require a validated licence irrespective of their strategic significance.

The Administration may be forced to make further concessions. Representative Bonker described the draft bill as "a sad day for American exporters" and said it was unacceptable in its present form. He wants to close a loophole that would let the president override the 270 day contractual grace period without declaring war or national emergency. At least seven alternative bills have been generated.

There is no sign of parallel concessions on the horizon for the scientific community, however. A report on scientific communication and national security published last October by the National Academy of Sciences (NAS) called for amendments in the existing act to exempt domestically available unclassified information and information not directly related to national security from the formal licensing procedure. These proposals have not been incorporated in the draft bill.

The omission may be simply a case of crossed bureaucratic wires. The NAS report was turned over to the White House Office of Science and Technology Policy (OSTP) last year and a response was expected last month. But the OSTP review was in turn subsumed in a larger and as yet unfinished inter-agency review of technological export being conducted by the National Security Council. An OSTP spokesman was unable to say whether the office had advised the Commerce Department on the contents of the draft bill.

If there is a less benign reason for the omission, however, the academy may find it difficult to make a late entry into a debate which has been dominated by the impact of the act on exporters rather than scientists. An NAS spokesman said last week: "We are concerned about it still and intend to see that the views of the NAS panel are taken into account in the debate on the Hill".

Peter David

Professional chemists beat inflation

ON average, British professional chemists will earn 8 per cent more in 1983 than in 1982 against an inflation rate of 5 per cent. This compares with the previous rise of 10 per cent between 1981 and 1982. The figures are taken from a survey carried out by the British Royal Society of Chemistry on their professional members, and refer to fellows and members of the society. These may be taken to represent professional

graduate chemists and biochemists with more than three years working experience. The licentiate members of the society earn, on average, 75 per cent of the members' and fellows' income, although the differential is around 10 per cent for the 25-34 year old bracket and increases to almost 40 per cent by 60-64 years. Source: *The Remuneration Survey 1983*, The Royal Society of Chemistry, £35.

Class of employment		25-29	30-34	35-39	40-44	45-49	50-54	55-59	60-64	Independent of age
Central government	LQ	7,400	9,130	10,920	11,650	14,290	14,280	14,290	—	10,970
	Med.	8,000	10,020	11,700	14,300	14,990	16,400	17,000	17,550	14,290
	UQ	9,370	11,200	13,210	16,010	18,500	19,930	19,730	—	17,080
UKAEA and associated Companies	LQ	—	9,810	11,750	—	—	13,000	—	—	11,440
	Med.	—	10,750	13,760	12,440	14,460	15,290	16,130	15,150	14,180
	UQ	—	13,430	15,650	—	—	18,340	—	—	16,120
Nationalized industry or public corporation	LQ	—	9,470	10,500	12,620	13,370	15,170	13,370	—	11,000
	Med.	9,390	11,000	12,880	14,200	15,950	17,300	18,190	17,810	13,910
	UQ	—	13,000	14,700	17,000	17,690	20,240	21,290	—	17,350
Local authority	LQ	7,650	8,700	10,000	10,920	12,140	11,540	11,950	12,160	10,400
	Med.	8,590	9,790	11,500	12,810	13,300	13,790	13,820	14,760	12,560
	UQ	9,400	11,020	12,810	14,110	15,490	15,730	16,000	18,870	14,680
University	LQ	7,470	8,500	11,550	13,500	14,940	15,970	16,180	16,180	13,500
	Med.	8,080	9,950	13,000	14,650	16,430	16,500	17,820	18,080	16,180
	UQ	8,730	11,500	14,150	17,100	17,740	18,500	21,500	23,000	17,830
Industry or commerce	LQ	8,000	9,280	10,700	11,700	13,000	13,250	13,700	13,040	10,600
	Med.	9,070	10,870	12,670	14,500	16,490	17,500	17,830	16,510	13,600
	UQ	10,510	12,850	15,450	18,000	20,330	22,950	24,000	21,000	17,800
Any other employer	LQ	—	8,340	9,800	10,210	11,240	10,170	11,750	—	9,860
	Med.	8,230	9,470	11,600	13,260	13,310	14,760	15,900	—	12,800
	UQ	—	10,480	13,100	18,080	17,850	19,810	21,500	—	15,500
Self-employed	LQ	—	—	—	13,500	9,000	12,000	8,000	10,000	11,000
	Med.	—	25,500	13,250	17,090	18,680	16,800	15,000	13,500	15,000
	UQ	—	—	—	41,340	29,000	23,650	26,000	22,000	25,000

LQ, Lower quartile average; Med., median; HQ, higher quartile average.

Sizewell inquiry

Funds for objectors growing well

FUND-RAISING appears to be going well for objectors to the plans of the Central Electricity Generating Board (CEGB) for Britain's first pressurized water reactor at Sizewell, Suffolk, despite the departure from the stage last week of the Sizewell B Appeal Fund (see *Nature* 24 March, p.282).

The fund closed with a total of £4,000 after approaches to industrial companies failed to attract support. Professor Sir Hans Kornberg, one of the fund's trustees, said he thought the fund had perhaps been too broad in its appeal, as most committed individuals would probably be supporting one or other of the main objectors directly. Among the major organizations being represented at the inquiry now being held at Snape in Suffolk, the Council for the Preservation of Rural England (CPRE) has already raised its initial target figure of £46,000 and is hoping to reach the £60,000 it now thinks will be needed. Friends of the Earth's "Sizewell Fighting Fund" has raised more than half its goal of £100,000 and the Town and Country Planning Association is well on the way to a target of around £50,000. The Stop Sizewell B Association, hopes to raise about £30,000.

Several county councils are also spending substantial sums on the inquiry. Northumberland County Council is

estimated to have spent £100,000. The Labour-controlled Greater London Council (GLC) is understood to have spent £50,000 on research for its presentation at the inquiry and is spending an additional £50,000 on publicity and conferences to air nuclear issues. Chief among these is the international conference on the urban transportation of irradiated fuel, which takes place in London this week.

The conference, organized by Technica, an independent firm of consulting scientists and engineers, attracted some influential participants, including Mr Christopher Audland, director general of energy at the EEC Commission, Mr Morris Rosen of the International Atomic Energy Authority and Mr Roger Clark of the UK National Radiological Protection Board. CEGB has, however, declined to send a representative: a board spokesman said GLC was already in possession of all available information on CEGB's fuel transport plans, and that CEGB felt it would be inappropriate to appear at a conference which was "clearly not impartial". Ms Patricia King-Smith, one of the conference organizers, says, however, that all shades of opinion are being represented, as GLC asked for an "unbiased conference".

Tim Beardsley

Romanian emigrants

Ceausescu's claw-back

ROMANIA's new emigration law, which demands that all citizens wishing to leave the country permanently must first repay the cost of their education, is an "internal matter" which does not contravene the Helsinki Final Act, according to President Nicolae Ceausescu.

The law, which was abruptly introduced last November (as a decree of the Council of State) has evoked sharp criticism abroad, particularly in West Germany and Israel — virtually all emigrants from Romania during the past few years have been ethnic Germans or Jews — and in the United States, where the State Department has said that it stands in the way of the renewal of Romania's "most favoured nation" status, due to expire in June.

During a visit to Austria last month, President Ceausescu told Alexander Wachsmuth, the Vienna correspondent of the West German news agency DPA, that the new regulations were simply a corollary of the existing Education Act of 1978, which obliges all graduates to work for a number of years in jobs assigned to them or otherwise to repay the cost of their tuition. Ceausescu said that he personally knew very few young people who were reluctant to take up the jobs assigned to them so that there were very few instances of fees having to be repaid. He has also said that the decree is not discriminatory in that it applies equally to those of German and Jewish origin.

In addition to the threatened loss of "most favoured nation" status, foreign criticism of the law could impede President Ceausescu's endeavours to act as mediator in the Lebanese war. As he told the Kuwait paper *As-Siyasah*, Romania "lives in the vicinity of the Middle East" and hence is directly concerned that peace should be established as soon as possible. But Israeli officials say that under present regulations, the "reunification of families" urged by the Helsinki Final Act can take place only if relatives abroad pay the education bills which, for a doctor of science, can exceed \$40,000.

In several respects the President's explanations seem somewhat ingenuous. The 1978 Education Act does indeed impose on school-leavers and students the duty to take up the posts to which they are assigned (Article 148/i) but education is guaranteed to be "free of charge, without payment of fees" (Article 126/a) and nothing is said about repayment of fees if a graduate does not go to the assigned job.

But if the new rules merely restate for intending emigrants the existing obligations on graduates, it is difficult to understand why there is not a sliding scale of rebates according to how many years the emigrant has worked in Romania.

Vera Rich

Tasmanian dam

State and government conflict

Canberra

A REGULATION published by the federal government making work on the Gordon-below-Franklin Dam in Tasmania illegal has precipitated an impending battle between state and federal governments in the high court. Drafted after the Tasmanian Premier, Mr Robin Gray, appeared deaf to two entreaties for a moratorium on work while a settlement was negotiated, the World Heritage (Western Tasmania Wilderness) Regulation states: "Except with the consent of the Minister (for Environment), a person (including Tasmania) shall not . . . construct a dam or associated works". Although offenders may be liable to be fined \$A5,000, enforcing the regulations presents formidable difficulties.

An intransigent Mr Gray, who has consistently turned down offers of compensation, has forced the federal government's hand. Apparently the non-negotiable bottom line that the dam must be stopped is unacceptable to Tasmania. As a consequence, both parties scurried to the high court on 6 April to seek injunctions — Mr Bob Hawke (Prime Minister of Australia) to enforce the regulations until the case can be heard and Mr Gray to challenge the validity of the new regulations. Nevertheless, since the regulations came into force

just before Easter, work has continued only outside the boundaries of the World Heritage site. This concession by Tasmania may not last if the matter is not resolved quickly. Yet several weeks may elapse before the waiting list for hearings in the high court is cleared.

At the core of the dispute is the power of the federal government to intervene in the affairs of state governments to uphold international treaties or conventions.

It is understood that the new federal regulation is an interim measure pending legislation to be introduced during the parliamentary sitting in May. Since the Australian Democrats holding the balance of power in the Senate have taken a strong anti-dam position, the measure is likely to have a smooth and speedy passage. On the face of it, however, if the present regulations are ruled invalid, legislation may fare no better because the same constitutional power is invoked in both cases. In that event, the federal government may have to resort to less direct means of achieving the desired goal, possibly by revoking the state's import and export licences or its authority to raise money overseas. Whatever the high court decision may be, Mr Gray has agreed to abide by it.

Vimala Sarma

US nuclear power

Reactor dispute near Brookhaven

Washington

LONG Island in New York, the home of the Brookhaven National Laboratory, contains one of the largest concentrations of nuclear scientists in the world. It also contains the Shoreham nuclear power plant, an 820-MW GE (General Electric) Mark 2 boiling water reactor which has cost more than \$3,000 million to build but may never be turned on because the municipality, Suffolk County, says the area is too congested to be evacuated safely in an emergency.

The county's objections may be the final straw for Shoreham's owners, the Long Island Lighting Company (LILCO). With construction delayed by protracted engineering problems and with huge cost overruns, Shoreham is already regarded as a case study in how not to build a nuclear power plant. Now it is about to become a legal test of whether local governments have, in effect, the right to veto the siting of nuclear reactors.

Construction of Shoreham began a decade ago and the company estimates that the plant will be ready to operate at full power next January, by which time its planned cost, and the price of the electricity it generates, will have multiplied many times. Before starting up, however, LILCO needs a federal licence to load the plant with fuel and begin low-power testing. Suffolk County has asked the Atomic Safety and Licensing Board of the Nuclear Regulatory Commission (NRC) to stop hearings on the licence application, on the grounds that without Suffolk's blessing the required emergency plan cannot be submitted for federal approval.

The licensing board is expected to make a decision next week, but its ruling is unlikely to settle the matter. LILCO contends that New York State can choose to submit a plan which bypasses Suffolk, and newly drafted federal regulations are sufficiently fuzzy to ensure that the board's decision will be challenged whichever way it goes.

Ostensibly, the impasse between LILCO and Suffolk is the result of a technical disagreement over the feasibility of coping with the aftermath of an accident at Shoreham. LILCO has produced an emergency plan, based on a 10-mile radius emergency planning zone (EPZ) around the plant, which envisages a total evacuation of the zone's population of under 200,000 within five to seven hours of an accident.

Suffolk's political leadership, after breaking off joint planning with LILCO and commissioning independent studies of the site hazards, has concluded that Long Island's congested access routes and rapidly shifting wind patterns call for a 20-mile radius EPZ, a zone which would contain more than 400,000 inhabitants. Such a zone could not possibly be

evacuated in time, the county believes.

Supporters of Shoreham, including many Brookhaven workers, accuse the county of fanning irrational fears about the plant for political purposes. As evidence of this, LILCO cites the county's abrupt decision a year ago to break off joint planning, a move that coincided with the arrival of a new county chief executive, Mr Peter Cohalan. Until then, the radiological emergency plan for Shoreham was being prepared by Suffolk's own expert, Dr Lee Koppelman, under a contract from LILCO. When Suffolk disowned Koppelman's plan and said it would ask independent experts to draft an alternative, LILCO submitted its plan to the state's Disaster Preparedness Commission (DPC) without the county's endorsement.

The Koppelman plan, based on a 10-mile EPZ, the minimum specified by NRC, has been refined by officials of New York's DPC and, it is believed, would have been forwarded for approval by the federal emergency management authority but for the insistence of New York Governor Mario Cuomo that LILCO and Suffolk settle their differences. Suffolk, meanwhile, had concluded that no safe evacuation plan was feasible for Shoreham and that the plant should therefore never be used.

Suffolk's position is based on several different technical studies. Robert Budnitz of Future Resources Inc. examined the probable accidents and the likely magnitude of a radiation release. F.C. Finlayson Associates undertook an analysis of the possible consequences, weather conditions and local population distribution. Neither study, according to Dr Vanz Sailor a Brookhaven physicist and Long Island resident, reached conclusions significantly different from the assumptions underlying the Koppelman plan. The Finlayson study concluded that only once in 500 million years would an accident occur requiring the evacuation of a 10 to 15 mile area.

The county did, however, attach considerable weight to studies of the "shadow effect" in an evacuation — the likelihood that residents beyond an evacuation zone would flee in the event of a nuclear accident and thereby overload the evacuation routes and emergency services.

Because the technical disagreements are so broad a reconciliation between LILCO and Suffolk is unlikely. What is more probable is a protracted legal dispute to determine whether, by refusing to cooperate on emergency planning, a local municipality can be allowed to kill off a reactor which has already been constructed.

Meanwhile, interest charges on Shoreham are accumulating at about \$1.5 million a day, costs which will be passed on eventually to the consumers of Long Island whether the reactor is allowed to operate or not.

Peter David

British telecommunications

Call collect

THE British Government seems to be moving briskly along a zigzag course towards its intended denationalization of British Telecommunications (or British Telecom), the electronic part of what used until recently to be known as the British Post Office.

The House of Commons agreed on 29 March by only a smallish majority (286 to 241) to the latest version of the government's bill, itself a far cry from the original prospectus introduced more than two years ago by Sir Keith Joseph the then Secretary of State for Industry. But the latest version of the bill still has to run the gauntlet of the House of Lords, which may mean that a recipe for denationalization cannot be complete until after the election.

Originally, the British Telecommunications Bill was intended merely to formalize the managerial division of the Post Office (which handles electronic written messages) and British Telecom (which handles electronic messages), but then to introduce a measure of private competition with the latter. The written message part has been uncontentionally dealt with.

Now, the chief objective of the bill is that there should be an executive officer of the British Government called the director-general of telecommunications who would supervise the provision of telecommunications in the United Kingdom, licensing potential providers when appropriate.

The latest version of the bill has the technical advantage of providing a relatively simple device by means of which shares in the existing public monopoly could be sold to members of the public. Eventually, the assets of British Telecom will be transferred to some new company or corporation, no longer enjoying a special status on taxation and able to market its own shares.

Two other issues have dominated the parliamentary debate so far — the fear that a private telecommunications monopoly will not provide services for rural users (voiced primarily by the government's supporters) and the fear that an open market in telecommunications will be a market for Japanese suppliers (chiefly shared by its opponents). Understandably, the government is requiring that the director-general should grant licences in a way that will cater for the rural voters.

The almost forgotten issue of when telephone users not buried in the country will be able to hook up to their telephone lines equipment available elsewhere in the world remains unclear. British Telecom and the Department of Industry have approved for general use three versions of a small-sized private branch exchange. The British Standards Institution, hopes to publish a general set of standards in July — a little later than the deadline originally fixed by the government for the passage of a final version of its bill. □

Sci-tech investment

New mutual funds signal healthy state of high technology stocks

Washington

BIOTECHNOLOGY stocks are, in the main, thriving (see below), and further proof of healthy market interest in science and technology stocks was provided at the beginning of the month by the launching of twin mutual funds which intend to invest in science and technology companies around the world. The funds, Sci/Tech Holdings Inc and Sci/Tech SA are notable for two reasons: the vast size of their operations and a novel plan to recruit leading scientists to an international scientific advisory council.

The funds started operating on 1 April with total net assets of \$835 million, the biggest sum of money ever in an initial public offering for the mutual fund industry. Sci/Tech Holdings Inc will be open to investors in the United States and Canada, Sci/Tech SA to investors elsewhere. Both funds will be managed

University; Chen Ning Yang, Nobel laureate and Albert Einstein professor of physics at the State University of New York, Stony Brook; Horton Stever, former director of the National Science Foundation and president of Carnegie Mellon University and Hideo Itokawa, president of the Systematic Engineering Research Institute.

Each member will receive \$2,500 a year in fees plus a similar amount for each meeting they attend formally, with an upper limit of \$7,500 a year. Dr Woolf will be paid an additional \$5,000. But these earnings will in fact be doubled since they are offered separately by each of the twin funds.

An initial statement by the funds' investment advisers include biotechnology and health care as areas with "superior" investment potential. Other areas included computers, communications, consumer electronics, electronic components and instruments, robotics and office and factory automation.

Peter David

Nature index of biotechnology stocks

BIAGEN NV, the company headed by Harvard biologist Walter Gilbert, saw little movement in the price of its shares after going public on 22 March. Its stock was offered at \$23 a share and rose a quarter of a point after 800,000 shares were traded on the first day. By last week its share price had fallen to 20¼.

Biogen's offering contrasted starkly with that of its competitor, Genentech, whose shares soared from \$35 to \$89 within hours of the company going public in 1980. But the contrast came as little surprise (see *Nature* 10 March, p.101). Had Biogen's shares remained at \$23 the market value of the company would have reached about \$430 million, close to 80 per cent of the present value of Genentech, still regarded by Wall Street as the benchmark company.

Market analysts are interpreting the sober reaction to Biogen as a sign that the feverish attitude Wall Street once held towards biotechnology companies has matured. The modest fall in its share prices is not being interpreted as a sign of lack of

confidence in Biogen, which plans to use the \$53.9 million it realized from the sale to switch from concentrating on research and development into testing and marketing its own products.

Among innovative biotechnology companies, Biogen remains nevertheless second only to Genentech in its scientific reputation. For both companies, and from a strictly commercial point of view, what will matter most in the next few delicate years of growth is whether it will be possible not merely to keep on spending on research but to sustain development as well. One snag, understood in other industries, is that development is about ten times as costly as research. Another is that the people who have made possible past growth will shrink from its application; having shown what should be put in bottles, they may be unwilling to spend ten times as much on telling how the bottles should be made. This is one reason why established companies may now find it easier to buy up smaller fry.



jointly by Merrill Lynch in New York, Lombard Odier in London and Nomura Capital Management in Tokyo.

A nine-member scientific advisory council, headed by Dr Harry Woolf, director of the Institute for Advanced Study in Princeton, will consult periodically with the investment managers and advise on trends in science. Members of the council include Bruce Hanay, former research vice president at the Bell Laboratories; Reimar Lust, president of Max Planck Gesellschaft; Frank Rauscher Jnr, senior research vice president of the American Cancer Society; Heinrich Ursprung, president of the Swiss Federal Institute of Technology; Itaru Watanabe, professor emeritus at the faculty of medicine at Keio

12-Month high	12-Month low	Company	Close previous month	Close 25 Mar.	Change
60¾*	16½	A.B. Fortia (Sweden)	52	58¼	+6¼
8	2	Bio-Logicals (Canada)	3¼	5½	+2¼
13	3¾	Bio-Response (USA)	11½	11	-½
16⅝	7¾	Cetus (USA)	14¼	15⅝	+1⅝
14½	6½	Collaborative Research (USA)	14½	13¼	-1¼
33½*	5¾	Damon (USA)	23½	33½*	+10¾
36¼*	8¼	Enzo-Biochem (USA)	30¼	34	+3¾
28	6⅝	Flow General (USA)	17¼	12⅝	-4⅝
69	26	Genentech (USA)	64½	54¾	-9¾
12½	2¼	Genetic Systems (USA)	10⅝	10½	-⅝
18¾*	7	Genex (USA)	17⅝	17½	-½
27¾	9⅝	Hybritech (USA)	27	22	-5
18½	5	Molecular Genetics (USA)	18½	17½	-1
27	8	Monoclonal Antibodies (USA)	21½	20½	-1
53¾*	34⅝	Novo Industri A/S (Den)	51⅝	51	-⅝

The *Nature* Biotechnology Stock Index for March 1983 stands at 200 compared with 195.1 last month. Base is 100 as of 25 June 1982. The previous index appeared in *Nature* 10 March, p.101. Close of month prices are the closing prices on the last Friday of each month. Yearly highs and lows are based on Friday trading prices. Where stocks are traded over the counter, the quoted is the bid price. Where stocks are traded on the American and New York stock exchanges, price quoted is the transaction price. Data from E.F. Hutton, Inc.

*New high or low for last 12-month period.

Darwin's finches worth a mention

SIR — In his report on the Linnean Society's discussion of evolution in the Galapagos, Peter Boag¹ said "Darwin... omitted any mention of the finches from his *Origin of Species*". While it is true that Darwin never specified this group either by its vernacular or its Latin name, he did nevertheless mention it several times in the *Origin*. To see this, it is necessary to remember that thirteen of the twenty-six species of land-bird known to Darwin from the Archipelago were Darwin's finches².

The relevant references in the *Origin* are few: I quote them in full from the first edition.

Page 48: "Many years ago, when comparing, and seeing others compare, the birds from the separate islands of the Galapagos Archipelago, both one with another, and with those from the American mainland, I was much struck how entirely vague and arbitrary is the distinction between species and varieties."

Page 390: "If we compare, for instance, the number of the endemic land-shells in Madeira, or of the endemic birds in the Galapagos Archipelago, with the number found on any continent, and then compare the area of the islands with that of the continent, we shall see that this is true."

Page 390: "Thus, in the Galapagos Islands, nearly every land-bird, but only two out of the eleven marine birds, are peculiar; and it is obvious that marine birds could arrive at these islands more easily than land-birds."

Page 397: "I will give only one, that of the Galapagos Archipelago, situated under the Equator, between 500 and 600 miles from the shores of South America. Here almost every product of the land and water bears the unmistakable stamp of the American continent. There are twenty-six land-birds and twenty-five of these are ranked by Mr Gould as distinct species, supposed to have been created here; yet the close affinity of most of these birds to American species in every character, in their habits, gestures, and the tone of voice, was manifest."

Page 400: "Thus the several islands of the Galapagos Archipelago are tenanted, as I have elsewhere shown, in a quite marvellous manner, by very closely related species; so that the inhabitants of each separate island, though mostly distinct, are related in an incomparably closer degree to each other than to the inhabitants of any other part of the world."

Darwin also has an implied mention of Darwin's finches in the essay of 1844³:

"The Galapagos Archipelago is a remarkable instance of this latter fact: here almost every bird, its one mammifer, its reptiles, land and sea shells, and even fish, are almost all peculiar and distinct species, not found in any other quarter of the world: so are the majority of its plants."

For the list of species of birds in the Archipelago, Darwin no doubt relied on the lists published in 1839 in the *Journal*², the first version of which is generally known as *The Voyage of the Beagle*. On page 461, he says, in listing the various bird taxa:

"9th. A group of finches, of which Mr Gould considers there are thirteen species; and these he has distributed into four new sub-genera. These birds are the most singular of any in the Archipelago. They all agree in many points; namely, in a peculiar structure of their bill, short tails, general form, and in their plumage. The females are grey or brown, but the old cocks jet-black. All the species, excepting two, feed in flocks on the ground, and have very similar habits. It is very remarkable that a nearly perfect gradation of structure in this one group can be traced in the form of the beak, from one exceeding in dimensions that of the largest gros-beak to another differing but little from that of a warbler."

In the much revised second edition of *The Voyage of the Beagle*, in 1845, Darwin illustrates four of the finches, and says:

"Seeing this gradation and diversity of structure in one small, intimately related group of birds, one might really fancy that from an original paucity of birds in this archipelago, one species had been taken and modified for different ends".

It is at least likely that Darwin, when writing the *Origin*, which he regarded as an abstract, thought that he had already given sufficient detail about Darwin's finches in *The Voyage of the Beagle*. As all these quotations show, Darwin was well aware of the importance of this famous group of birds.

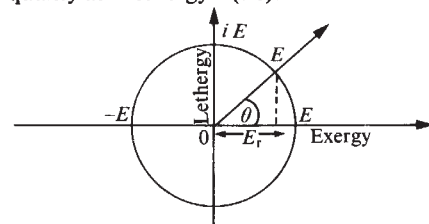
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Lethergy derived

SIR — Exergy (= useful or available energy) is a directed number = (E, θ) — the "Exergy vector". The directed number $(1, \frac{1}{2}\pi)$ is, as usual, denoted by i where $i^2 = -1$. Exergy is a complex number with real (x) and imaginary (iy) components. The useful component of exergy is the real x -axis ($E_r, 0$) component. As imaginary constituents are added the x -axis component is reduced. Where only the imaginary component ($E, \pi/2$) or (iy) exists, that is, the exergy vector lies along the y or imaginary axis, we define the quality as "lethergy" (sic).



Lethergy is not therefore mere absence of exergy but a positive force leading in the wrong (imaginary) direction. It can, in theological terms, be likened to evil — which is not absence of good but a positive malevolent force leading in the wrong direction. In other words, imaginary solutions to the energy equation lead in the wrong direction, reducing the amount of exergy or useful energy available.

(The concept can be generalized in industrial terms with the real axis as industry and the imaginary axis "sloth", giving the industry-sloth diagram for economic analysis.)

The lethergy or (iy) component is a function of the constraints on the energy supply system.

Constraints which increase the lethergy

component of the exergy vector can be listed:

- (1) Lack of investment
- (2) Long engineering lead times
- (3) Management inexperience
- (4) Union proliferation and internal strife
- (5) Lack of political will to initiate long term strategy
- (6) The Environmental lobby
- (7) Over-optimism about technological solutions
- (8) Raw material shortages
- (9) Emotional paralysis — fear of a nuclear accident
- (10) Planning delay and impotence
- (11) Lack of trained manpower and construction potential

In extreme instances the angle of the exergy vector (E, θ) can exceed $\pi/2$ so that the projection onto the x -axis becomes negative. For example, the Austrian decision, based on a referendum, not to proceed with or use nuclear power after building the Zwentendorf nuclear station resulted in a negative exergy component.

Where $\theta > \pi/2$, energy policy can be seen to be having a negative effect.

Some other solutions have a strong "imaginary" or "lethergy" component of the exergy vector. For example:

(i) Solutions with large renewable or solar energy components. (ii) High technology solutions still requiring research and development, for example fusion.

On the other hand North Sea oil and gas and improved technology (for example, fuel-efficient cars) make an important "real" contribution to the exergy vector.

IAN FELLIS

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Dodson's choice

SIR — The biology of the archaebacteria is currently exciting much interest and among leading contributors to our understanding of this bizarre group of organisms is W. Zillig (see ref. 1). Before a new terminology develops to confuse interested onlookers, (otherwise known as "borogroves")² it is perhaps worth recalling that, give or take a consonant or two, Lewis Carroll², with remarkable foresight, seems to have had the matter taped. . .

" 'Twas Zillig, and the slithy toves

Did gyre and gimble in the wabe; . . . "

Surely, "slithy toves" are none other than archaebacteria and "wabes" their "solfataric water holes". How long will it be before "gyre-ases" and "gimble-ases", the enzymes responsible for their extremely thermophilic metabolism, are described?

D.H. LEWIS

University of Sheffield, UK

1. Fisher, F., Zillig, W., Stetter, K.O. & Schreiber, G. *Nature* 301, 511 (1983).
2. Carroll, L. *Through the Looking Glass* (Macmillan, London, 1872).

Worries about infectious cancer

The discovery in the past two years that a specific virus may be responsible for many T-cell malignancies raises awkward questions for blood banks — and the rest of us.

CAN people catch cancer, as they catch influenza? Strictly speaking but in a strictly limited sense, the answer so far is that they can. The best attested evidence that viruses can cause cancer by person-to-person transmission is a kind of exception to what might be expected to be the rule: infection with hepatitis B virus usually causes a kind of jaundice but appears occasionally to cause liver cancer as well. The well known association between the occurrence of Burkitt's lymphoma in central West Africa and the presence of Epstein-Barr virus in the tissues of those affected also suggests the transmission of a specific agent from one person to another, but the evidence is only circumstantial.

The human T-cell lymphoma leukaemia virus, known as HTLV, is a more plausible specific agent of person-to-person transmission of a specific malignancy. Since the discovery of the virus three years ago by Dr Robert C. Gallo and his associates at the US National Institutes of Health, geographical associations suggestive of person-to-person transmission have been revealed, notably in south-west Japan. The latest contribution from Gallo's laboratory (see page 626) can only strengthen the opinion that HTLV is indeed an infectious virus.

Gallo and his associates have surveyed some dozens of people, some of them with lymphoid disease and some without, looking both for the presence in the lymphoid genome of nucleotide sequences characteristic of virus DNA and for antibodies against the viral antigens, among which the protein p24 is the best known. The results are striking. T cells from patients with the form of T-cell malignancy known as adult T-cell leukaemia (ATL) almost always contain HTLV sequences. Most but not all of these patients have HTLV antibodies as well — the exceptions seem to be cases in which the full virus has not been integrated into the genome and is thus defective. There is also evidence (from one patient only) of the occurrence of HTLV antibodies in a healthy person whose cells lack the DNA sequences characteristic of the viral genome, suggesting an infection headed off.

Thus, in a sense, a wheel has turned full circle. Three decades or so ago, when it first became clear that particles resembling viruses could be found in malignant tissues in humans (not merely animals), oncologists naturally expected that it would be possible to apply the well tried techniques

of vaccination to the protection of people against cancer. By and large, that hope was disappointed. And even now, too little is known of the biology of cancer-related viruses to know why. Indeed, recent discoveries have made the role of viruses in malignancy even more confusing. For if, as in the case of the T24 bladder carcinoma, the difference between the genome of a normal and a malignant cell is a point mutation at one site in a putative oncogene (see, for example, Tabin *et al.* *Nature* **300**, 143; 1982), but if the oncogene is also apparently identical with one gene of a retrovirus, there is no simple way of telling whether the aberration has arisen by chance or by a kind of dead-end infection whose effect is merely that a retrovirus has delivered the aberrant gene to an infected cell which is itself incapable of regenerating viruses. None of this diminishes the importance of the discovery that particular oncogenes are associated with particular kinds of cancer, or that the oncogenes may be simple variants of naturally occurring genes. But it may be some time before causation is better understood.

HTLV is clearly different. The circumstantial evidence is strong that transmission between people does occur, although the mode of transmission can only be guessed at this stage. And it is now plain that infection provokes a response from the immune system. So vaccination is in principle on the cards. The practical consequences are nevertheless far from clear. For while the T-cell lymphoid diseases are distressing and usually fatal, they are not so common as to constitute a serious problem of public health. At least in industrialized societies, the incidence of lymphoid malignancies of all kinds among adults is typically only a few in 100,000 a year, and even in south-west Japan, where HTLV infection was first recognized to be unusually common, and where up to 25 per cent of the population in a coastal strip of Kiyushu may have antibodies against HTLV, the overt incidence of ATL is, nevertheless, only ten times greater than elsewhere. In these circumstances, even if vaccination turns out to be feasible (which is likely) the costs may exceed the benefits. Interrupting the mechanism of transmission, which appears to require intimate contact — perhaps sexual, perhaps infant dependence on maternal milk — may make more sense.

More immediate questions arise, however. Whatever the natural mechanism of HTLV transmission, on the face of

things there is a high risk that the transfusion of blood from an infected person to another will transmit HTLV. So here, as with hepatitis B virus, is another headache for the blood transfusion services which have become essential accompaniments of public health services in many (but still too few) parts of the world. Will it now be necessary to ensure not merely that blood donors are free from familiar contaminants such as hepatitis B but from HTLV as well?

The ethical dilemma is plain. Avoiding transfusion with contaminated blood should be a public duty for the transfusion services. Even as things are, screening blood against the risk of hepatitis B relies to a large extent on the willingness (and ability) of donors to give truthful accounts of circumstances in their backgrounds that may have exposed them to infection. Given the long latency of ATL (measured in decades), and the risk that a donor from a population in which HTLV is known to be relatively frequent may carry the virus in a still cryptic form, there seems no sure alternative to the application of techniques such as those used by Gallo and his colleagues to all suspect samples.

The problem is likely to be further complicated when more is known of geographical and ethnic distribution of the disease. Gallo's survey, now published, demonstrates the occurrence of HTLV not merely in patients with T-cell malignancy from south-west Japan but in others from Israel, the Caribbean and the United States. The chances are that, as with hepatitis B, populations from some of the poorest parts of the world will turn out to be the most common carriers of the virus. So here again, as with the fuss in the United States some years ago about proposals to identify carriers of the sickle-cell gene, the transfusion services are likely to find themselves grappling with the awkward political problems of saddling some ethnic groups with the slur of being potential carriers of a serious infection. In the circumstances, the best hope is that what is now being learned about HTLV will be used quickly to eliminate infection by HTLV and thus, in due course, to eradicate T-cell malignancy as a whole. □

Thanks are due to Robin Weiss (Institute of Cancer Research), W Blattner (National Cancer Institute), Beatrice Hahn (National Institutes of Health) and Mel Greaves (Imperial Cancer Research Fund).

Archaeology

Tectonic movement and agrarian collapse in prehispanic Peru

from David L. Browman

PERUVIAN farmers on the north coast of Peru are able to use only 60–65 per cent of the land that prehistoric populations had under cultivation. Standard explanations, including erosion, salinization or desertification because of poor land management, prove to be incorrect. Instead collapse appears to have resulted from the impossibility of maintaining the agrarian canal system in the face of tectonic uplift, with resultant downcutting and entrenchment of the hydrological regime, and episodic regional catastrophies.

New evidence to support this view comes from two related studies of prehistoric 12th century Chimú culture irrigation networks in the Moche River valley. The first study deals with the functioning of the 70 km long Chicama–Moche Intervalley Canal (or La Cumbre Canal) (Fig. 1). Over the last decade, four separate teams have worked on this canal under the direction of Michael Moseley of the Field Museum of Natural History, Chicago. Their interpretations were initially contradictory: Farrington¹ argued that the canal carried water along its entire length and that its subsequent collapse was due to an engineering miscalculation; the Pozorskis² maintained that the canal never carried any water because the Chimú (AD 1000–1470) lacked the necessary engineering and surveying capabilities to complete it; Kus³ believed that the canal carried water for part of its length but had never been intended to carry water from one valley to the other and is best conceived as a massive make-work project; and Ortloff, Feldman and Moseley⁴ believed that the Chimú were master canal builders but the system was rendered inoperative by tectonic slope changes during the period of construction.

The principle fact leading to these divergent opinions is that in at least seven locations, the canal now runs uphill for a kilometre or more. But does it run uphill because of some ancient engineering or surveying error, or does it only run uphill because of tectonic tilt-induced slope change after the original construction? If

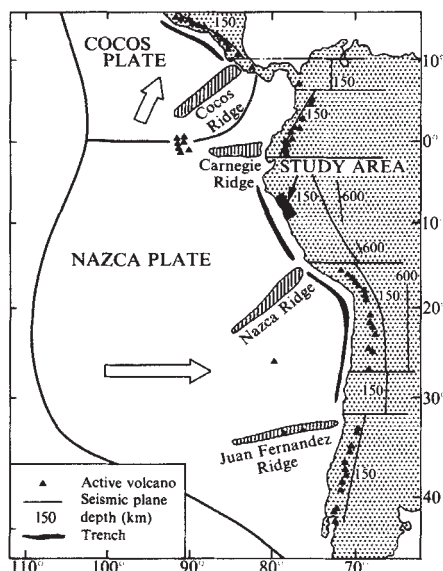


Fig. 2 Plates and aseismic ridges in the eastern Pacific and study area. Adopted from *Mosaic* 11, 1980.

we can find evidence of engineering expertise and of the continuous functioning of the canal beyond an apparent uphill slope interval, then tectonic movement must surely provide the explanation.

The Chimú clearly had the ability to design and build the intervalley canal. Before intervalley canal construction was

begun, they had been successfully designing, building and maintaining canals for 2,000 years. Chimú canals in the Moche valley that existed at the same time as the intervalley canal were aligned with accuracies of less than 0.25° over considerable lengths, and construction techniques indicated a level of understanding of the complex relations between wall roughness, bed slope, hydraulic radius, subcriticality and supercriticality of flow rate not known until the late 19th century in Europe and America^{4,5}. These abilities make it very unlikely that repeated engineering errors — seven uphill sections — would be made on the intervalley canal.

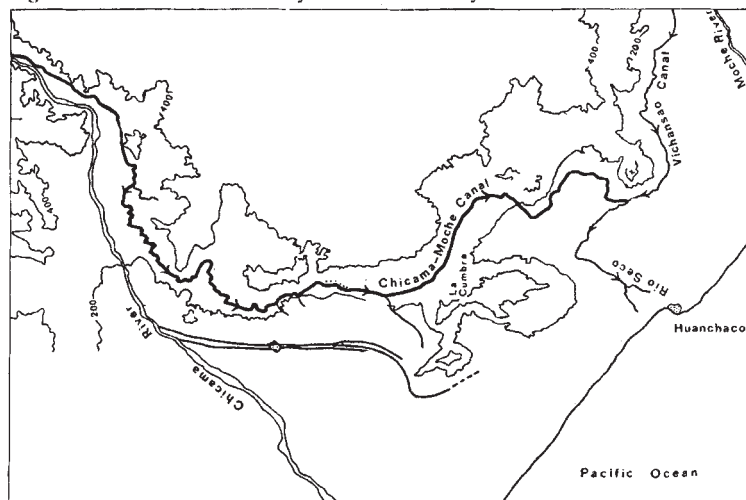
There is agreement that the canal functioned for half its length, from the canal intake at the Chicama River south to a point near the divide between the two river drainages. This portion includes at least three long sections where water would now have to run uphill. This evidence, plus similar observations on sections of the Cerro Portachuelo canal on Pampa Cacique on the south side of the Moche valley⁶ which has similar uphill slopes but was known to have functioned, indicate that tectonic uplift made the canals impossible to use after they had been built.

Changes in ground slope could result from the interaction of the subducting Nazca Plate with the South American Continental Plate (Fig. 2). Forces induced by subduction act on a network of coastal subplates delineated by fault lines paralleling the Andean cordillera and intersecting with a series of transverse fault lines coincident with the rivers in the north coast valleys⁷. The Nazca Plate is subducting at an average velocity of 10 cm yr⁻¹, producing 1–2 cm aseismic vertical crustal movement per year⁴.

Planes of crustal movement induce slope distortion in the intervalley canal. The fault scarp at the intervalley divide has assumed a high flexure configuration because the cordillera is rising faster inland than it is along the coast⁸. Therefore tectonic displacement is increasing the slope of the scarp faster than it is lifting the landscape crossed by the canal course⁹. The major intervalley active fault occurs at the intervalley divide, where the channel of the canal lies directly atop a bedrock block that has been upthrown at the fault, with a slope tilt upward to the south. Ortloff, Moseley and Feldman argue that this large perturbation in the slope of the intervalley canal is the result of tectonism, not engineering error. Their associates find it hard to believe that aseismic uplift could produce a 70 m displacement, and are more taken with arguments that this uphill section fossilizes evidence of sloppy engineering^{1–3}.

Kus has put forth an intriguing alternative hypothesis. He notes that modern canals in the Moche and Chicama valleys

Fig. 1 Chicama and Moche valleys and the intervalley canal.



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which have slopes and construction comparable with the prehistoric canals can lose more than 5 per cent of their water per km, due to seepage and evaporation. Over the 70 km length of the intervalley canal, this loss could result in a net delivery efficiency of as little as 3 per cent. Kus thus argues that the canal was never practical as a mover of water. Instead he feels the canal can only be understood socially, as a massive make-work project³ by the Chimu rulers, in response to tectonically induced reduction of available irrigation water from the Moche River itself. Radiocarbon dating indicates the canal was constructed between AD 1050–1100 and AD 1250. Moseley⁵ estimates that it would have taken 5,000 men working 8 h days for 6 months a year (between growing seasons) 100 years to construct the canal.

The tectonic hypothesis is, however, further supported by analysis of the serial contraction of the canal systems within the Moche valley. Three physical factors affected these canals: encroaching dune fields, rare episodic floods and gradual tectonic uplift; but the agrarian collapse in the valley appears most closely tied to tectonic uplift. Uplift will cause river entrenchment and water table subsidence. As rivers entrench the efficiency of intake by the canals will fall. The archaeological evidence shows that intakes were adjusted as the rivers cut deeper into the landscape but the intakes were eventually stranded when they could no longer be moved further upstream or cut deeper because of bedrock. In the Moche valley a cycle of abandonment of canals and construction of new intakes further down river can be seen, resulting in a contraction of farming towards the river and a retreat downvalley of irrigation as it became increasingly difficult to move water laterally to the fields. The land irrigated began decreasing not from lack of water, but from the impossibility of delivering the water because of tectonic uplift.

The gradual collapse brought about by tectonic uplift was punctuated by environmental catastrophes as well as by episodes of radical uplift. Minor incursions of the 'El Niño', associated with shifts in the Equatorial Counter Current, can be observed once every 15–16 yr. More severe

Fig. 3 The Chimu built massive aqueducts to carry their canals over low areas. One of the rare torrential rains washed out this aqueduct, exposing an earlier channel (behind the person) buried beneath the visible one. Such large-scale rebuildings of the canals were necessary because tectonically induced ground slope changes rendered earlier canals inoperative.



incursions occur on average once a century, and there are rare major catastrophic floods once every 600–800 yr.

Three major catastrophic floods have been identified. The Moche area is a desert, receiving less than 2 mm of rain a year, but in 1925, 395 mm fell, with 226 mm falling in a three-day period, and caused a major multi-valley north coast flood with destruction of canals and settlements. Moseley and associates¹⁰ found evidence of a second catastrophic flood around AD 1100 which was at least twice as large as the 1925 flood, with flood waters at least 18 m deep in the central portion of the valley. Sheet wash erosion destroyed large parts of the irrigation system, many of which were not subsequently rebuilt. Earlier still, before AD 700, the Moche valley appears to have experienced an even larger flood⁵. Such events have little effect on the potential for irrigation, but have a major effect on the actual systems and the capabilities of the local people to sustain agriculture.

Canals, being linear features, are not the most appropriate remains measuring strike and dip, and rotation of the tectonic uplift. Ortloff⁷ has just begun a study of architectural features to check ground slope alterations. Work on two structures at the site of Chotuna, in the Lambayeque valley about 175 km north of the Moche valley, indicate

uplifting towards the south along the N–S axis, with less movement along the E–W axis. A gradual plate tilt in the N–S direction with the southern part higher is consistent with the agricultural field and canal data from the Moche valley, and is also consistent with the observations of lithospheric plate movement, which is greater in the south near the Chilean frontier and which displays vertical displacement decreasing from south to north⁶. The study of prehistoric monumental architecture may thus allow a time-angular motion history of the coastal plates on which the sites ride. Analysis of the data is still being carried out, but preliminary measurements by Ortloff⁷ suggest a N–S uplift of 1° every 550–800 yr.

Tectonically induced tilt has led to a rate of loss of arable land in the Moche valley of 25 per cent each 1,000 yr (refs 5, 10). At the moment the Peruvian government is making large investments in major reclamation projects on the north coast. To avoid these projects meeting the same fate as the Chicama–Moche Canal it is vitally important to understand why the prehistoric system collapsed and to take into account forces such as tectonic uplift which have previously been thought to work on too slow a time scale to seriously affect construction activities.



Fig. 4 View of the excavated Chicama–Moche Intervalley Canal. In some places the canal was cut through rock, in others terraces over 35 m high were built of stone and earth.

1. Farrington, I. *Am. Antiq.* 48(2) (in the press); Farrington, I. & Park, C. C. *J. arch. Sci.* 5, 225 (1978); Farrington, I. *Am. Indígena* 40(4), 691 (1980).
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10. Nials, F. *et al. Bull. FMNH* 50(7), 4; 50(8), 4 (1979). Ortloff, C. in *La Tecnología en el Mundo Andino* (eds Lechman, H. & Soldi, A.) 91 (Univ. Naco. Auto. de Mex., Mexico DF, 1981).

The Burgess shale

Strange animals from the Cambrian seas

from Richard A. Fortey

THE fossil record is usually very imperfect. Only those animals and plants with preservable hard parts are candidates for fossilization, and of these only those which lived in favourable environments, such as the continental shelves, are likely to have a complete record. The result is that our picture of past life in the sea tends to be somewhat biased. Reconstructions of the sea bed in popular books or in museum displays often show the sea floor of the Palaeozoic swarming with brachiopods and trilobites, but leave out the worms and other soft-bodied animals that were likely to have been just as numerous.

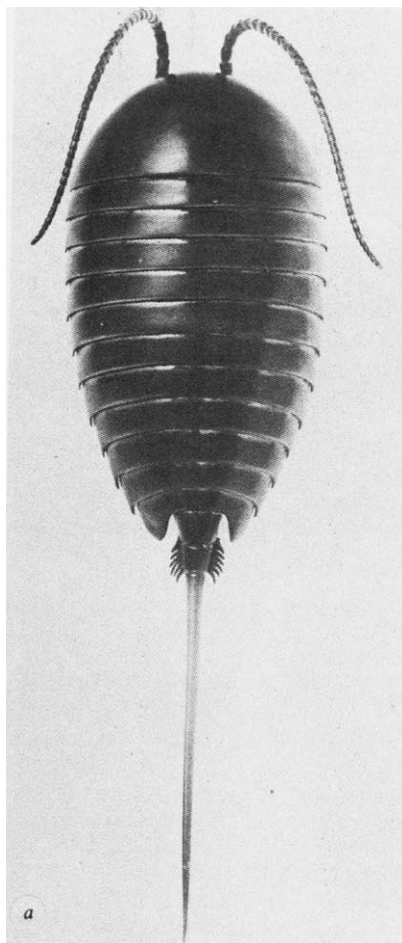
The rare occurrences where the whole fauna has been preserved are of great importance in correcting this biased view. Soft-bodied animals are only capable of preservation under special circumstances — where the bodies could be buried rapidly without decay, for example. One of the earliest of these is the Middle Cambrian (530–550 Myr) Burgess Shale of British Columbia, a fauna originally discovered in 1909, but which has recently been intensively re-collected and studied by H.B. Whittington of Cambridge University and his co-workers.

The Burgess Shale animals originally lived on muddy sediments at the sloping foot of a submarine cliff. The sea bottom was not stable, and from time to time great slumps occurred, carrying sediments and animals alike down the slope, to come to rest in a small basin. The rapidity of this burial is shown in the fossils because they are preserved almost higgledy-piggledy — some buried head first, others on their sides — and even the same species can present very different appearances depending on its orientation.

More than fifty species of soft-bodied animals occur in the Burgess Shale. The majority are arthropods — presumably distant relatives of living crustacea and horseshoe crabs — but there are several kinds of annelid and priapulid worms, and there is also a solitary representative of our own Phylum Chordata. Other strange animals defy inclusion in any group of organisms living in the sea today. Together they prove how rich and varied was the fauna even at this early time. The arthropods in particular show an amazing array of adaptations and most of these, too, are difficult to relate to living species.

The latest of the bizarre animals to be described (D.L. Bruton & H.B. Whittington *Phil. Trans. R. Soc. B300*, 553; 1983) are two arthropods: *Emeraldella* and

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Leanchoilia, illustrated here. So exquisite is the preservation that A. Jensen of the Palaeontological Museum in Oslo has been able to build detailed models of the animals. No other fossils of like antiquity

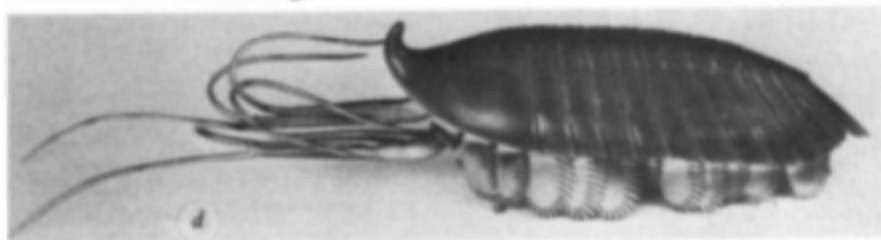


have been reconstructed in such detail.

But even this complete knowledge does not help in resolving the relationships of these animals to the living fauna: Bruton and Whittington conclude that *Emeraldella* is related to one other animal — and that, too, known only from the Burgess Shale. *Leanchoilia*, with its extraordinary 'great appendage' on the head, may also have relatives in the Burgess fauna, but its living relatives are unknown. This is usually explained by describing the animals at this remote time as being at some kind of 'experimental' stage in their development, but whatever the explanation it is proof of the flexibility of the arthropod design, at a time when the insects, living exemplars of arthropodan ingenuity, were still more than 100 million years in the future.



All illustrations are taken from Bruton and Whittington (*Phil. Trans. R. Soc. B300*, 553; 1983). a, Dorsal view of model of *Emeraldella*. b, *Emeraldella* (approx. $\times 2$). c, *Leanchoilia*, head end (approx. $\times 1.5$). d, Left lateral view of model of *Leanchoilia* (approx. max. known size).



Archaeology

A palaeolithic treasure house in the Pyrenees

from Paul G. Bahn

THE Volp caverns in the foothills of the French Pyrenees, 65 km south of Toulouse, have been famous since the early part of this century because of the remarkable wall decoration in the cave of Trois Frères and the engravings and clay bison in the Tuc d'Audoubert. Both caves are in an outstanding state of preservation thanks to the care of three generations of the Bégouën family, the proprietors¹, and constitute two of the very rare intact magdalenian sites which can provide a wide range of information on man in the late ice age².

Both contain evidence of brief occupation, and the Tuc also has foot- and heel-prints, and evidence of children having played in the clay near the sculptures. An in-depth study was made recently of the numerous objects brought into the caves or moved around by man³: they range from flint tools to animal teeth, and many were carefully placed in wall fissures. These items — some exotic, some banal — appear deliberately placed, often in close proximity to particular parietal figures or to significant features of the caves. Details of this type — missed or dismissed as unimportant by earlier scholars, both here and in other sites — are providing new insights into the behaviour and beliefs of the caves' occupants.

The third Volp cave, that of Enlène, is equally important, but far less famous owing to its complete lack of parietal decoration. The whole of this large cave was occupied by Magdalenians of the same period as the others; it is joined to Trois Frères by a passage which was also used by the occupants, since it is rich in portable art, and other, objects. Enlène is one of the richest magdalenian sites in France, but this was not generally realized because of the repeated and unmethodical plundering that it suffered from the 1860s onwards. Excavation by the Bégouëns in the early part of this century was of a higher standard, but was abandoned in the 1930s when it was realized that the methods employed were outdated. The recently

resumed excavations⁴ have brought a full range of modern techniques to the site, with spectacular results.

Investigation has been concentrated in the cave's depths, up to 180 m from the entrance. One part of the work involved removal of the many spoilheaps from previous excavations; these have been wet-

maintained under a stalagmite in the 'Salle des Morts'. It yielded a first stratigraphy, two hearths and a broken perforated baton decorated with a salmon⁵; the hooked lower mandible reveals it to be an exhausted male salmon in the autumn, a subject which has been found in other Pyrenean magdalenian sites. One of the hearths contained an

abundance of burnt bone, perhaps used as fuel, which gave a radiocarbon date of 11,990 BC. Removal of previous spoilheaps in this *salle* revealed a total of 11 such hearths: burnt, bowl-shaped depressions in the cave floor, some containing calcinated bones, others none.

In the 'Salle du Fond', 15 m² of intact deposits have been found under the spoilheaps. Excavation has uncovered several interesting features as well as a living-floor with a dense scatter of bones and flints: for example, a large fragment of stalagmite floor had been set upright and wedged into place with small stone slabs⁶; and an enigmatic line of bone fragments had been pushed vertically into the clay floor.

The cave contains literally thousands of 'plaquettes', small slabs of sandstone, schist and so on, which were brought in by the occupants from a source 200 m from the entrance. They are 2–3 cm thick, up to 15 × 11 cm in size; most are fragmented, many carry traces of burning (even when not found in or near hearths) and some are engraved. Earlier discoveries led to the theory that the slabs may have been used as lamps, and that much of the breakage was deliberate and therefore ritual⁷: fragments of particular engravings have been found widely separated,

sometimes with patination or calcite on the breaks. It was also observed that the decorated examples had their engravings face-down, and this was thought to be another aspect of ritual. Similar burnt broken engraved slabs were found in the Tuc, and other deep Pyrenean magdalenian caves such as Labastide, Isturitz and the Mas d'Azil. Many examples were found around hearths, and indeed were

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Sandstone slab from the cave of Enlène (Ariège, France) engraved around 12,000 BC (size, one-tenth of original). The central portion, discovered in the 1930s, is in the Musée de l'Homme, Paris. The two others, discovered in 1980, are in the Bégouën collection. The engravings depict the lower part of a bison, a human 'couple' and a third anthropomorph. Photograph reproduced by permission of Count Robert Bégouën and Jean Clottes.

sieved and have produced a huge amount of stone tools, bones and art objects. All the magdalenian material of Enlène, whether disturbed or *in situ*, is homogeneous, from dense but relatively short-lived occupations. The presence of tens of thousands of flint flakes and of numerous nuclei shows that much toolmaking took place in the cave itself.

An intact square metre of deposit re-

sometimes used in their construction. As for the engravings, only the finest realistic examples were published, although it was noted that hundreds bore undecipherable or abstract traces⁸; Breuil noticed that bison heads were a very common motif⁹, although horses, deer, fish, birds and even humans were also drawn. At both Labastide and Enlène an engraved slab was set upright in clay, like a *stela*.

The renewed work in Enlène has revealed a 5 m² area paved with slabs of this type. The many burnt *plaquettes* are still interpreted as possible lamps, although a theory has also been put forward that they represent a kind of 'hot-plate' or heating device¹⁰; sandstone has thermal qualities and a resistance to tension which make it suitable for domestic functions of this type.

The 'face-down' rule seems to have lost its validity: some examples are decorated on both sides and it is obvious that engravings are far less likely to remain visible on the upper face which was eventually covered with a patina or with stalagmite. Indeed, a great number of engravings are amazingly difficult to see, even on pristine surfaces: the earlier discoverers saw that many drawings were exceedingly fine scratches, barely visible to the naked eye, but it is the recent campaign in Enlène which has revealed the sheer numbers of engraved examples. Apparently virgin slabs can no longer be tossed aside; every one has to be examined very closely. No less than 574 engraved specimens have so far been found at Enlène, many of them in the spoilheaps of earlier workers.

Engraved stones are common in many magdalenian sites, but the quantity at Enlène places it alongside such important sites as Limeuil, La Marche and Gönnersdorf. The Magdalenians in these and other places appear to have devoted a great deal of time and effort to engraving lines and pictures onto stone slabs.

The theory of deliberate breakage has found further support at Enlène, where an as yet unpublished engraving of an anthropomorph seems to have been carefully decapitated. The slab pictured on the previous page deserves particular mention: the central portion was discovered in the 1930s and interpreted as a bison-leg and

two humans⁸. The two new fragments were found, 1.5 m apart, in 1980, and fit the first perfectly; they confirm the interpretation, and complete the two humans and add a third elsewhere. The bison was engraved first, the 'couple' later: it is assumed that they represent a male and female, though the only evidence lies in their comparative robustness/gracility and the long hair of the 'female'¹¹. If so, it is just possible that

the scene represents a copulation, a subject of extreme rarity in palaeolithic iconography. It is to be hoped that further fragments will be found in the intact portion of the Salle du Fond as excavation progresses. □

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Transport mechanisms

The biology of the wheel

from Jared Diamond

MOST inventions that have proved useful in human vehicular transport were anticipated by nature. Animals and plants had invented controlled combustion, powered flight, gliders, submarines, jet propulsion, parachutes and missiles long before humans did. Why did evolution not anticipate the most important element of human transport, the wheel? Why do rats not roll on wheels but run on feet, and why are there no propeller-driven fish? The answer to this seemingly frivolous question is more complicated than one might at first think.

Almost all our land vehicles are wheeled, and many of our sea and air vehicles are driven by propellers. These and other rotating systems have two key virtues for transport: they substitute rolling resistance for sliding resistance and their kinetic energy is constant rather than fluctuating. The table illustrates these virtues by comparing energetic costs of transport for vehicles living and non-living. The most efficient known vehicle is a human on a bicycle, requiring only one-fifth of the energy of the same human walking². Even paraplegics in wheelchairs are 24 per cent

more efficient than healthy humans walking, despite using arm muscles rather than the stronger leg muscles and having the extra weight of the wheelchair³.

Other comparisons in the table illustrate that transport costs decrease with vehicle weight, are lower for swimming than for flying, lower for flying than for locomotion on land, independent of number of legs, and higher for motor vehicles than for animals of the same weight and transport mode⁴⁻⁶. Thus, the only vehicle likely to prove more efficient than a cyclist is a whale or large fish.

The table suggests that animals would have been better off if they had evolved the wheel. Conventional wisdom answers that this was impossible, because blood vessels and nerves cannot pass through a rotating joint to supply nutrients and commands to a living wheel¹. This rule seemingly derives support from an exception: the flagella of bacteria⁷⁻¹⁰ and some unicellular eukaryotes^{11,12}, which provide the sole biological instances of a true wheel and axle. Unlike most eukaryote flagella, which beat sinusoidally within a plane, these exceptional flagella propel their living vehicle

Cost of transport

Vehicle or animal	Weight (g)	Transport mode	Wheels?	Cost*
Cockroach	5	Walk	—	65
Mouse	22	Walk	—	40
Land crab	150	Walk	—	25
Bee	0.1	Fly	—	14
Rabbit	2,500	Walk	—	4.5
Hummingbird	3	Fly	—	4
Helicopter (Sikorsky S62)	4,000,000	Fly	+	3.5
Parrot	35	Fly	—	2.5
Small jet plane (F105)	20,000,000	Fly	+	1.5
Dog	10,000	Walk	—	1.5
Small propeller plane	1,000,000	Fly	+	1.2
Sheep	30,000	Walk	—	1
Pigeon	384	Fly	—	0.95
Automobile (Cadillac)	2,200,000	'Walk'	+	0.8
Human walking	70,000	Walk	—	0.75
Large jet plane (DC8)	100,000,000	Fly	+	0.6
Horse	600,000	Walk	—	0.6
Human in wheelchair	80,000	'Walk'	+	0.57
Salmon	50	Swim	—	0.4
Human on bicycle	80,000	'Walk'	+	0.15

Data are from refs 2-6.

*Calories per gram per kilometre

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by rotating. This can be demonstrated by tethering the flagella to glass, whereupon the bacterium^{7,8} or algal cell¹² itself can be seen to rotate. In the protozoan *Cryptotermes cavifrons*, the head and axostyle rotate continuously clockwise with respect to the cell body¹³. These unicellular organisms are so small that diffusion suffices to supply nutrients to the flagellum or axostyle through the rotating joint. For larger animals diffusion would be inadequate.

Reflection suggests, however, that problems of nutrient supply to larger living wheels cannot be the sole reason for their non-existence. Countless animals bear other non-metabolizing external structures without blood and nerve supplies, such as claws, hair, scales, exoskeletons and some horns. Why have animals not evolved non-vascularized wheels and propellers as well? Paws might be more useful than wheels in some situations, but cats' claws are retractable; why not retractable wheels? For the purposes of further discussion, let us evaluate a hypothetical rat with retractable roller-skates on its paws, abbreviated RRR.

A recent paper by LaBarbera¹⁴ approaches the RRR problem from a direction opposite to conventional wisdom, by examining why animals should not, rather than could not, evolve wheels. For land transport LaBarbera analyses three severe limitations on the wheel's utility, arising from a terrain's compliance, vertical irregularity and horizontal irregularity.

Driving on our asphalted roads, we overlook the problems of terrain compliance faced by RRRs in natural environments, until our car gets stuck in mud or sand. Rolling resistance increases with surface compliance, which is about 10 times higher for sand than for concrete^{15,16}. The rolling resistance ratio for a soft surface compared with a hard surface increases with vehicle weight. For a given vehicle weight the ratio decreases with wheel diameter. For a given wheel diameter the ratio decreases with wheel width, but the effect is negligible for large-diameter wheels. Thus, considerations of terrain compliance select against roller-skated elephants and favour evolution of wide-rimmed large-diameter wheels for roller-skated rats on soft terrain. The tires of dirt bikes and dune buggies reflect these considerations, which also explain why the energetic advantages of wheelchairs over walking disappear on carpets¹⁷ and why government regulations aimed to make buildings accessible to the handicapped require any carpets to be firm and unpadding.

The second limitation that LaBarbera analyses for wheeled vehicles on land stems from vertical obstacles. The highest obstacle that a wheeled vehicle can surmount is about one-quarter to one-half of the wheel radius for a vehicle unable to shift its centre of gravity¹⁵. Even if the centre of gravity can be shifted, as one would expect to be within the capabilities of RRRs

and other hypothetical wheeled animals, the limiting obstacle height equals the wheel radius. The frequency of vertical irregularities in natural terrain is inversely proportional to wavelength: low obstacles are far commoner than high ones^{15,16}. This consideration dooms wheeled ants in most habitats and frustrates human cyclists and wheelchair-users at curbs.

The third limitation to wheeled vehicles on land comes from obstacles in the horizontal plane. The turning radius of a rolling wheeled vehicle depends in a complex way on wheel size, number and position and on the length and width of the wheelbase. Without doing the calculations, one can see intuitively that RRRs would have their roller skates retracted most of the time in most vegetated habitats.

These considerations suggest that wheeled terrestrial animals and plants are most likely to evolve on open flat hard-packed terrain. LaBarbera points out several biological examples convergent on wheels. Dung beetles (Scarabaeinae) of African savannahs feed themselves and their larvae on dung balls which range in diameter from 4 mm to 5 cm and which they roll at speeds of up to 23 cm s⁻¹ for up to 10 m (ref.18). The already discussed limitations imposed by vertical obstacles may explain why no dung beetles roll dung balls smaller than 4 mm. Seed dispersal in the plants known as tumbleweeds occurs as a result of the whole plant or its infructescence being rolled long distances along the ground by strong ground winds^{19,20}. This dispersal mechanism has evolved independently in many plants of deserts, prairies and open shores. Self-propelled rolling is employed by several animals, including rolling spiders, somer-

saulting shrimp²¹ and rolling pangolins. For example, the Malayan pangolin *Manis javanica*, a large lightly armoured good-tasting mammal, often escapes predators by curling into a ball and rolling down steep hills²².

The three limitations on wheel utility for terrestrial animals do not apply in the sea. Why, then, are there no propeller-driven fish or birds? LaBarbera points out that efficiencies for converting input power to thrust are only about 60 per cent for ship propellers, up to 90 per cent for typical airplane propellers and 88 per cent for the ingenious propeller of the *Gossamer Condor*. However, an oscillating flexible foil, analogous to a fish's tail and possibly to a bird's wing, can reach efficiencies of 96–98 per cent²³. Large fish actually achieve these values. Thus, the puzzle is not why fish failed to evolve propellers, but why engineers failed to evolve oscillating flexible foils.

Even for humans on land, the utility of the wheel in transportation is limited by the same three considerations relevant to animals and plants. Modern civilization runs on wheels, but mainly for long trips and mainly on prepared surfaces designed to be of low compliance, vertically smooth and horizontally uncluttered. Self-propelled wheeled vehicles have been familiar to us for a long time: the bicycle since 1816, the roller-skate since the early 1700s, the wheelchair since 1655. Yet even the most urbanized human prefers walking to roller-skating as s/he circulates in buildings, in gardens and on the sidewalk.

There are also two famous cases of highly civilized, politically centralized societies that had large draft animals and still found it more efficient to dispense with wheels for transport. One case is the absence of wheeled transport from the New World before Columbus, even from areas of South America that used llamas as beasts of burden. Astonished Europeans concluded that Amerindians had simply never thought of the wheel, but this view is refuted by the discovery of pre-Columbian pots illustrating wheeled carts used as toys.

The other case involves the success of the pack camel at completely replacing wheeled transport in all North Africa and the Middle East from Morocco to Afghanistan about 1,500 years ago, following the invention of the North Arabian camel saddle²⁴. The explanation for this voluntary abandonment of wheeled vehicles is that the pack camel was more cost-efficient than a wagon drawn by ox, horse, or camel — even neglecting the capital expense of building and maintaining roads for wagons. Wheels continued to be used in North Africa and the Middle East for irrigation, pottery making and mills.

In short, animals and the transportation systems of many human societies lack the wheel, not because they could not evolve or invent it, but because they are better off without it. □

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Nucleic acid structure

New twists to left-handed DNA

from Stephen Neidle

THAT DNA appeared a relatively monotonous molecule in structural terms was perhaps an inevitable outcome of the classical Watson-Crick model, for it seemed that the regularity of the anti-parallel right-handed double-helix imparted a precise structural equivalence to all residues in a DNA sequence. The past three years have, however, seen profound changes in our view of nucleic acid structure, and a more complex and subtle picture is emerging. Of particular interest has been the discovery of the quite unexpected left-handed Z-DNA helix. Crystallographic studies of d(CpG)_n oligonucleotides^{1,2} with $n=2,3$ and fibre diffraction analysis of poly(dC-dG) (ref. 3) first revealed this profound structural deviation from the classical picture, and ready correlations have been found in a stream of studies of Z-DNA structure and reactivity, in solution (see *News and Views* 292, 292; 1981), the latest installment of which is reported in this week's *Nature* (see p.632).

In early studies on the Z structure in solution poly(dC-dG) was mostly used as it readily displays a salt-induced right- to left-handed helix transition. Wells and his colleagues⁴ pioneered the insertion of varying lengths of d(CpG)_n tracts into recombinant plasmids, and showed them to be remarkably sensitive and versatile probes of Z-DNA properties, especially when embedded in natural right-handed DNA sequences. A closed-circular DNA plasmid is negatively supercoiled when it contains normal right-handed helical DNA but when parts are converted to a left-handed form by the insertion of d(CpG) there is a loss of superhelicity which can be readily monitored by changes in either superhelical density or electrophoretic mobility. The inserted d(CpG)_n segments have to be substantially longer than 10 base pairs (possible more than 20) for this relaxation of negative supercoiling to occur⁵; but as the process is energetically favourable, it can occur at a much lower salt concentration than that required for the B→Z transition in linear poly(dC-dG) (refs 6,7) and may indeed be driven by torsional forces alone.

Recent experiments have dispelled any lingering doubts over whether the relaxed d(CpG)_n plasmid really contains left-handed DNA. The use of antibodies raised against Z-DNA has shown that the antibody combining site for a CG-modified pLP32 plasmid is clearly at the insertion site⁸.

Z-helix formation appears not to be confined to d(CpG)_n sequences — Arnott *et al.*³ showed that poly(dG-dT)·poly(dA-dC) could equally well adopt a left-handed Z

structure in a semi-crystalline fibre sample. However, Wells *et al.* have found⁹ that this polynucleotide will only form a left-handed helix, in the most extreme environmental conditions. Furthermore, they were unable to observe the transition with an almost perfect d(TpG)₃₁ sequence either alone or when inserted into a plasmid. These findings are important since such a 62 bp sequence has been found in a eukaryotic DNA, from the 3' side of the mouse immunoglobulin α gene. If Z-DNA does indeed have a biological role, in, say, gene regulation and signalling, one might expect that such a sequence would readily adopt a left-handed form.

The controversy concerning the Z-DNA potential of d(TpG)_n sequences is further fuelled by a report in this week's *Nature* (p.632). Haniford and Pulleyblank used a fundamental property of closed-circular DNAs containing a left-handed region embedded within a normal right-handed helix — the degree of supercoiling (and hence the linking number) is altered compared with the all-right-handed form. A mixture of such DNAs was constructed from the plasmid pDPL6 by cloning varying lengths of d(TpG)_n sequence into it. These modified plasmids (topoisomers) had differing degrees of supercoiling, depending on the amount of left-handedness introduced. Gel electrophoresis of both the topoisomers and unmodified plasmids after the same degree of partial supercoil relaxing had been applied suggested an abrupt structural transition in the d(TpG)_n region after a certain degree of unwinding had been produced in the plasmid as a whole.

Detailed examination of a plasmid with a 60 bp d(TpG) insert showed that the superhelical DNA turns in it were unwound to roughly twice the extent of sections of DNA sequence with hairpin-loops. The torsional free energy for initiation of this B→Z transition was calculated to be quite high, almost twice that required for stabilization of the Z form, and possibly higher than that required for d(CpG)_n sequences. The previous failure to observe Z-DNA with d(TpG)_n inserts⁹ may thus be due to the use of a plasmid with a superhelical density insufficient for the initiation of the transition, suggesting this sequence is less readily driven to the Z form than the d(CpG)_n one. There is little doubt that this difference originates in differences in the water molecules and ionic environments surrounding these sequences compared with natural DNA; but a full explanation is not yet possible.

The continuing search for a definite biological role for Z-DNA has recently revealed some potentially significant fin-

dings. Following the location of regions in *Drosophila* polytene chromosomes that react with Z-DNA antibodies¹⁰, at least four specific Z-DNA-binding proteins, have been detected in *Drosophila*¹¹, using affinity chromatography. The proteins not only interact with, but actually stabilize Z-DNA sequences; such proteins would enable d(TpG)_n sequences to flip from right- to left-handed helices rather more readily than do simple salts. Thus, polyarginine stabilizes the poly(dC-dG)·poly(dC)·dG Z helix at physiological ionic strengths¹², rather than the > 2.5 molar salt concentration normally required.

At the more detailed sequence level two recent reports using specific hybridization probes have revealed that potential Z-forming d(TpG)_n sequences in eukaryotic DNA are widespread. Such results do not, however, prove that these regions are in, or can indeed change to, a Z-form helix. First, Hamada and Kakunaga¹³ have found a d(TpG)₂₅ region in an intron of a human cardiac muscle actin gene. Use of their d(TgG)_n-specific hybridization probe on restriction fragments of human DNA revealed a very large number of such sequences — an estimated 10⁵ copies — in the genome. This result is, in retrospect, not altogether surprising, in view of the large amount of Z-DNA banding found previously in *Drosophila*¹⁰.

Second, Walmsley *et al.*¹⁴ have examined the genome of *S. cerevisiae* yeast with a d(TpG)_n hybridization probe. Their finding of at least 30 regions of d(TpG)_n is in itself not surprising but they then went on to isolate the 1.4 kilobase chromosome ends (telomeres), which themselves hybridized with the putative Z-DNA probe. Furthermore, the telomeres, when cloned with the linear plasmid having chromosomal ends from the protozoa *Tetrahymena*, retained hybridization properties, which were carried through after restriction enzyme cutting of the plasmid into separate *Tetrahymena* and yeast telomere-containing fragments. They conclude that both these eukaryotic telomeres contain d(TpG)_n tracts, which, if one accepts that these are involved in regulatory processes, implies features common to these very different eukaryotes, at least for the initiation of replication at chromosomal ends. [1]

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Somatic generation of antibody diversity

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In the genome of a germ-line cell, the genetic information for an immunoglobulin polypeptide chain is contained in multiple gene segments scattered along a chromosome. During the development of bone marrow-derived lymphocytes, these gene segments are assembled by recombination which leads to the formation of a complete gene. In addition, mutations are somatically introduced at a high rate into the amino-terminal region. Both somatic recombination and mutation contribute greatly to an increase in the diversity of antibody synthesized by a single organism.

A CENTRAL part in the immune system is played by antibodies which can recognize and distinguish specific molecular patterns of antigens. Because antigens are diverse in structure, the repertoire of antibodies must be large and is estimated to be at least one million and probably one to two orders of magnitude higher¹⁻⁴. The way in which the genetic information required to code for this exceedingly large number of different but related proteins is stored in the genome and transmitted through generations has been the central issue of immunogenetics during the past few decades.

The basic unit of an antibody, or immunoglobulin (Ig), molecule is composed of two identical light (L) chains and two identical heavy (H) chains (Fig. 1). Extensive sequence analysis of many immunoglobulin chains revealed that the amino-terminal regions are highly variable while the carboxy-terminal regions are one of several types within a given species^{5,6}. The variable (V) region is primarily responsible for antigen recognition and contains particularly variable subregions whose residues have been implicated in actual antigen contact (ref. 7; also see ref. 8 for a review). These subregions, which are referred to as complementarity-determining regions (CDR1, CDR2 and CDR3), are flanked by less variable subregions termed framework regions (FR1, FR2, FR3 and FR4). The constant (C) regions are responsible for a variety of effector functions such as complement fixation and Fc receptor binding and define the chain types or classes (Fig. 1).

The idea that the V and C regions of an immunoglobulin chain are encoded by two separate 'genes' in germ-line cells which undergo a rearrangement for joining in lymphocyte development was first suggested by Dreyer and Bennet⁹. This hypothesis was an attempt to accommodate two seemingly contradictory observations: (1) the extreme heterogeneity in the primary structure of the V regions within an individual animal and (2) the observation that an allotypic determinant (a serologically identifiable determinant commonly present on immunoglobulin chains of a particular type or class) segregates as a single mendelian trait¹⁰. The former result suggests that each individual carries a large number of structural genes for immunoglobulin chains while the latter result indicates that there may be only one gene for each of the several chain types or classes.

Thanks to restriction enzymes and recombinant DNA technology, the enigma of immunoglobulin genes has now been resolved at least in outline. It was found that an immunoglobulin polypeptide chain is encoded in multiple gene segments scattered along a chromosome of the germ-line genome¹¹⁻¹⁶. These gene segments must be brought together to form a complete immunoglobulin gene active in B lymphocytes^{11,16-24}. In addition, mutations are introduced somatically into an immunoglobulin gene at a high rate^{17,19,25}. Both the recombination and mutation greatly diversify the genetic information carried in the germ-line genome. What follows is a brief review of these somatic events in mice. Recent studies on other mammalian

systems have confirmed the universality of the basic principles²⁶⁻²⁹.

Organization of immunoglobulin gene sequences

Mouse immunoglobulin chains are encoded in three unlinked gene families: λ light-chain genes, κ light-chain genes and heavy-chain genes, residing on chromosomes 16 (ref. 30), 6 (refs 31, 32) and 12 (refs 31, 33, 34), respectively. In mice, λ chains form only about 5% of the total serum immunoglobulin light chains and are much less heterogeneous than κ chains or heavy chains. Thus, it is no coincidence that the first complete description of the sequence organization of an immunoglobulin chain gene was made for the major λ chain subtype of mouse, namely $\lambda 1$ chains (see Fig. 1 legend for the definition of λ

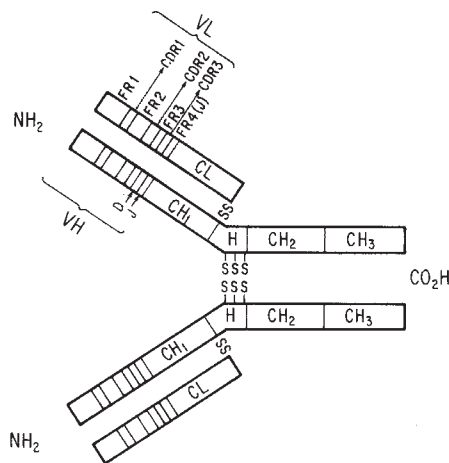
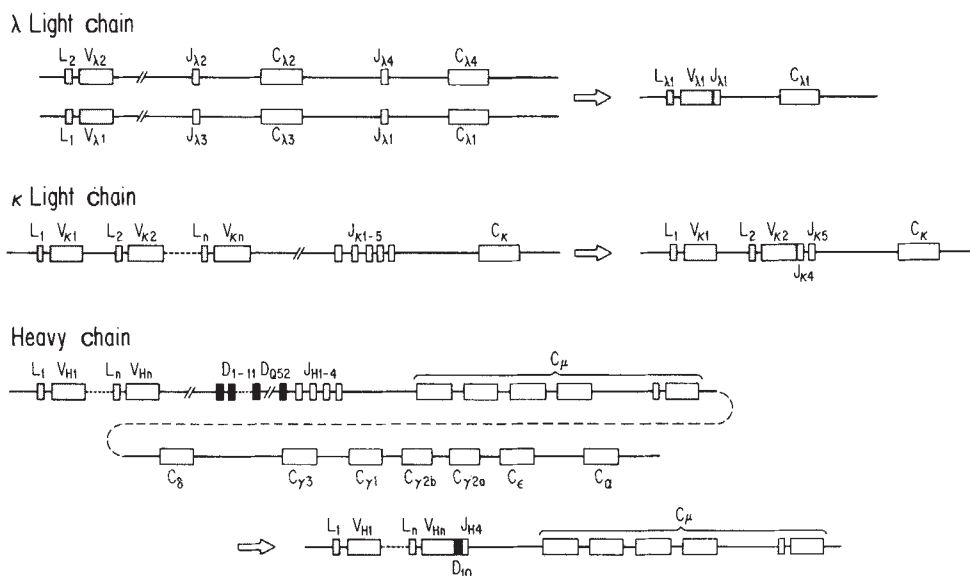


Fig. 1 Basic structure of a mouse IgG molecule. An immunoglobulin molecule is composed of two identical light chains and two identical heavy chains, each of which consists of the amino-terminal V region (V_L or V_H) and the carboxy-terminal C region (C_L or C_H). The V region contains amino acids primarily responsible for the antigen recognition which are enriched in the three complementarity-determining regions, CDR1, CDR2 and CDR3. The rest of the V regions are referred to as framework regions, FR1, FR2, FR3 and FR4. FR4 forms almost all of the J region, while the core portion of the heavy chain CDR3 is referred to as the D region. The C region executes a variety of effector functions. The light chains appear in two types, κ and λ . The mouse λ chains are further subdivided into three subtypes, $\lambda 1$, $\lambda 2$ and $\lambda 3$. Each light-chain type or subtype is defined by a unique C region sequence. Mouse immunoglobulin molecules are conventionally grouped into several classes and subclasses defined by specific C_H region sequences. In the mouse, eight C_H regions, μ , δ , $\gamma 3$, $\gamma 2a$, $\gamma 1$, $\gamma 2b$, ϵ and α define as many classes or subclasses, IgM, IgD, etc. The C_H region is composed of three or four internal homology units C_{H1} , C_{H2} , etc. In addition, a cysteine-rich hinge (H) region resides between C_{H1} and C_{H2} of γ chains.

Fig. 2 Organization of various mouse immunoglobulin gene segments. The organization of various immunoglobulin gene segments before and after somatic rearrangement (to the left and right of the arrows, respectively) is shown. The rearranged state illustrated is only one of many possible cases in each gene family. The double slashes indicate that the distance between and the relative orientation of the two flanking sets of gene segments have not yet been determined. Within each set of gene segments connected by a solid line, the relative position of each gene segment has been determined and the orientation of all segments is identical reading 5' to 3' from left to right. The broken lines in the V_K and V_H segment clusters indicate that the exact number and the relative orientation of the V -gene segments in each cluster are unknown. However, in a few cases studied, the relative positions of two or more adjacent V segments have been determined and their orientation has been shown to be identical (refs 55, 115 and G. Heinrich, unpublished). The broken line in the D -segment cluster and that between the C_μ - and C_δ -coding exons are only for illustrative purposes and the distance and the relative orientation of the involved gene segments have been determined. D_1 to D_{11} represent the D segments belonging to the SP2 and FL16 families (see ref. 53). The nine D segments of the SP2 family form a cluster spanning about 60 kb. The two D segments belonging to the FL16 family are located in the 5' end region of the SP2- D cluster. The 12th D segment, D_{Q52} , has been mapped 696 bp 5' to the J_{H1} segment⁵¹. The orientation of the SP2-FL16 D cluster relative to and its distance from the V_H cluster or the D_{Q52} - J_H - C_H cluster are unknown. However, the fact that all of these D segments are deleted in many myelomas and Abelson virus-transformed B cells harbouring V-D-J rearrangements on both copies of chromosome 12 suggests that the D cluster is located between the V_H cluster and the D_{Q52} - J_H - C_H cluster. Likewise, it has been argued that the L_1V_1 segments are 5' to the $J_3C_3J_1C_1$ unit and the L_2V_2 segments 5' to the $J_2C_2J_4C_4$ unit^{36,39}. The intron-exon structure of C_H segments other than the C_μ segments are abbreviated.



subtypes). In the germ line or most other cells which do not make a $\lambda 1$ light chain, the coding potential of this polypeptide chain lies in three separate DNA segments referred to as V , J and C ^{17,19}. (I shall denote DNA segments in italics and the corresponding regions of the polypeptide chain in roman type.) The coding potential of the major portion of the signal peptide is in a fourth DNA segment, L . The V segment codes for the rest of the signal peptide and the ~95 amino-terminal residues (numbering of amino acids is according to Kabat *et al.*³⁵) which compose the classical V region minus most of FR4, which is encoded by the J segment. The C segment codes for the entire C region. The L and V segments are separated by a 93-base pair long intron, I_1 , and the J and C segments by a 1,150-bp long intron, I_2 . The V and J segments are separated by a spacer of an unknown length. In the B lineage cell in which a $\lambda 1$ light-chain gene is expressed, the spacer between the V and J segments has been removed so that V and J are fused tail to head^{17,19}. Thus, a complete $\lambda 1$ chain gene consists of three exons and two introns arranged in the following order: 5' L_1I_1VJ, I_2, C 3'.

Recently, the analysis has been extended to the genes coding for $\lambda 2$ and $\lambda 3$ subtype chains (Fig. 2). The λ gene family contains four pairs of J and C segments, J_1C_1 , J_2C_2 and J_3C_3 , corresponding to the three known λ subtypes, and J_4C_4 which is defective³⁶⁻⁴⁰. It also contains two pairs of L and V segments, L_1V_1 (ref. 19) and L_2V_2 (refs 14, 41). The V_1 segments are preferentially joined with either J_3 or J_1 and the V_2 segment with J_2 (refs 42-44), although the preference is not absolute (refs 39, 45, also B. Blomberg and H. Eisen, personal communication).

Studies on the κ and heavy-chain genes have confirmed that an immunoglobulin gene sequence is split in the germ-line genome and that their gene segments are reorganized during B-cell development^{11,15,16,18,20-24}. However, the details of the sequence organization are somewhat different in each gene family, reflecting the degree of heterogeneity of the three families of polypeptide chains. Thus, in contrast to the λ loci,

the haploid genome contains a single copy of C_κ segment^{18,46,47}, a cluster of five homologous but different J_κ segments located from 4.0 to 2.5 kilobases (kb) 5' to the C_κ segment^{21,23} (the middle J_κ segment, $J_{\kappa 3}$, is defective), and a cluster of 90-300 different V_κ DNA segments⁴⁸, each of which carries its own L_κ segment (Fig. 2). The significance, if any, of the difference in the relative organization of J and C segments in the κ and λ loci is unclear. The V_κ - J_κ joining occurs in various different combinations among the κ chain-synthesizing clones of B cells to form complete κ -chain genes with a variety of V_κ regions^{18,20-23,49}.

In the heavy chains, the core portion of the CDR3 composed of 1-13 residues is encoded by a DNA segment referred to as D , which occupies an independent position in the germ-line genome^{22,24,50-53}. The haploid germ-line genome (Fig. 2) contains a cluster of 100-200 different L_H - V_H segments^{54,55}, a cluster of about 12 D segments⁵⁰⁻⁵³ (see Fig. 3), a cluster of 4 functional J_H segments^{22,24,56} and a cluster of 8 copies of C_H segments, one each for the eight immunoglobulin classes or subclasses⁵⁷⁻⁶⁰. Each of the eight C_H -coding sequences is composed of multiple exons, each of which corresponds to an internal homology unit forming a structural domain^{61,62}. In addition, the hinge regions of γ chains⁶¹ and the intramembrane and cytoplasmic portions of all eight chains are encoded by independent exons^{59,63-67}.

In the cells in which a heavy-chain gene is expressed, one each of the V_H segments, D segments and J_H segments are joined tail to head to form a complete V_H region-coding DNA segment (Fig. 2)^{22,24}. Because the V_H region is encoded in three gene segments and the joinings can occur in various combinations, the diversity that can be potentially generated by rearrangement is much greater for the heavy chains than for the light chains. The heavy-chain gene that is first formed is the one for the μ chain which defines IgM, the class of immunoglobulin that appears first in B-cell development among eight classes or subclasses. Although not directly related to the present subject, namely the diversity in the antibody recognition

Fig. 3 Nucleotide sequences of germ-line *D* segments and *D* regions of complete heavy-chain genes. On the basis of sequence homology, the 10 germ-line *D* segments sequenced to date can be classified into three families, FL16, SP2 and Q52. In addition, two more *D* segments thought to belong to the SP2 family have been identified but have not been sequenced⁵³. Of the 16 somatic *D* sequences from the rearranged, complete heavy-chain genes expressed in myelomas or hybridomas that have been characterized by DNA sequencing, 13 can be assigned to one of the three germ-line *D* families based on the sequence homology. The other three somatic *D* sequences, those of myelomas H76, HOPC1 and M173, do not show any obvious homology to any of the identified germ-line *D* sequences and characteristically contain stretches of G-C pairs. In fact, even among the homologous 13 somatic *D* sequences, none corresponds exactly to any of the germ-line *D* sequences. However, two lines of argument suggest that the repertoire of germ-line *D* segments is not much greater than the known 12. First, if additional germ-line *D* segments existed which corresponded exactly to the somatic *D* sequences, at least some of them should have been detected by the probes used because these probes cross-hybridized with less homologous *D* segments⁵². Second, in myelomas, hybridomas and other B and T lineage lymphocytes, joined *D-J* segments thought to be intermediates of *V-D-J* joining have been found on the allelic chromosome 12^{51-53,78,79}. All eight randomly selected *D-J* joinings used 1 of the 12 known germ-line *D* segments and *D*_{Q52} and *D*_{FL16.1} occur twice each. This suggests that the total repertoire of germ-line *D* segments should not be statistically much greater than the known 12. To determine the boundaries between *V_H* and *D* as well as between *D* and *J_H* in the assembled gene, the nucleotide sequences of three regions, namely the 3' end of the germ-line *V_H* gene, the assembled *V_H-D-J_H* and the 5' end of the germ-line *J_H* segment, were compared. For M141 (ref. 22), anti-PC myelomas (S107, M603, M167)²⁴ and anti-NP hybridomas (B1-8, S43)¹⁰⁵, these three sequences are available. The nucleotide sequences in the 3' ends of these three different germ-line *V* segments are highly conserved. Assuming the other germ-line *V* segments have the same degree of sequence homology, the *V_H-D* boundaries of the complete genes were deduced for those *V* genes whose germ-line segments have not yet been sequenced, namely MPC11¹¹¹, H76¹¹², *V_H*²¹¹³, *V_{H49}* (ref. 113), MC101¹¹⁴ and 'M173'⁵² and B38¹¹⁵. *D* sequences of H76, HOPC1 and M173 cannot be classified into any of these families. The sequence of *D*_{FL16.1}, or *D*_{SP2.3}, or *D*_{Q52} is used as the reference sequence of each family for comparison with the others. A dash indicates that the nucleotide in question is the same as that of the reference sequence.

FL16 FAMILY		SP2 FAMILY	
Germ line		Germ line	
D _{FL16.1}	TTTATTACTACGGTAGTAGCTAC	D _{SP2.3} , D _{SP2.4}	TCTACTATGGTTACGAC
D _{FL16.2}	--C-----CTAC	D _{SP2.5}	-----A--T--
Myeloma, hybridoma		D _{SP2.6}	C-----
S107	-----	D _{SP2.7}	C-----A--T--
M603	-----C	D _{SP2.8}	C--G-----A--T--
M167	GG-----A-----TTTG	D _{SP2.2}	-----A-----
MPC11	GGAT-----AA-----CC	Myeloma, hybridoma	
B1-8	TACG-----	M21	--GG--A--T--CC
MC101	AGGG-----T-----G--GACCC	B38	C--G-----A--T--GGGG
M141	CGTCTCAA-----C-----G--AAATACCTCACTT	Q52 FAMILY	
V _{H2}	--A-----	Germ line	
V _{H49}	-----TC	D _{Q52}	CAACTGGGAC
'M173'	TGCCCC-C-C-----A--TAC--GGGGGT	Hybridoma	
H76	GC-GGGGGTCCCC	S43	TAT-G-----GG
HOPC1	GGGGAACCCCAT		
M173	AGCCCC		

sites, the expression of the other heavy chains (that is, δ , α , and so on) for the other class or subclass immunoglobulins requires additional sequence reorganization that takes place either at the level of DNA (switch recombination)^{22,68-71} or during the processing of the primary RNA transcript^{59,66,67,72}. For the reason given above, I shall not discuss them further.

Heptamer-nonamer and 12/23-base pair spacer signal

The mechanism of *V-J* or *V-D-J* joining is not known and the main possibilities will be discussed in more detail below. There are, however, specific sequences that seem to act as joining signals.

All germ-line *V* and *D* DNA segments are followed by either a heptamer, CACAGTG, or its analogue and a nonamer, ACA₅C₂, or its analogue separated by a short spacer of an apparently unconserved sequence (Fig. 4). Likewise, all germ-line *D* and *J* segments studied to date are immediately preceded by a consensus nonamer, G₂T₅GT, and a consensus heptamer, CACTGTG, separated by a spacer^{21-24,51,52}. Note that the heptameric and nonameric sequences following a gene segment (for example, *V_K*, *V_H* or *D*) are complementary to those preceding the gene segment (in this case *J_K*, *D* and *J_H*, respectively) with which it recombines^{21,23}. In addition, the heptamers are palindromic around the central A-T or T-A pair.

All *V_K*, *J_K* and *D* spacers are 12 bp long, while almost all *J_K*, *V_K*, *V_H* and *J_H* spacers are 23 ± 1 bp long (one exception is the apparently nonfunctional *J_{K3}* whose spacer is 21 bp long) (see Fig. 4)^{22,24,51,52}. Note that functionally meaningful recombinations occur only between two gene segments, one having a 12-bp spacer and the other a 23 ± 1-bp spacer.

It has been postulated that the recombinase contains two DNA-binding proteins, one recognizing the heptamer and nonamer with a 12-bp spacer and the other recognizing them with a 23 ± 1-bp spacer^{22,24}. The complementarity of the two pairs of the signal sequence may merely reflect the subunit

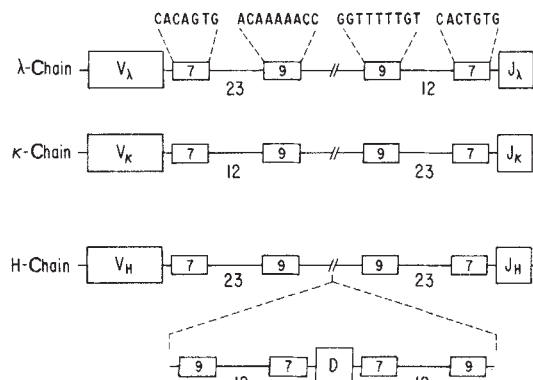


Fig. 4 Heptamer-nonamer and 12/23-bp spacer rule. See text for explanation.

structure of the recombinase. Alternatively, with the aid of the putative DNA-binding proteins, the two heptamers might form transient base pairs which will juxtapose the two recombining sequences^{21,23}.

Imprecise joining ends

The joining end(s) of an immunoglobulin gene segment is (are) imprecise and this contributes to antibody diversity. In κ chains, the amino acid position 96, which is usually encoded by the first *J_K* triplet, is a hot spot^{49,35}. At this position, most κ chains carry one of the four amino acid residues that can be fully encoded by one of the four germ-line *J_K* segments^{21,23,73}. However, the 96th codons of some of the rest of the κ chains could be generated if we assume that only the second and third bases or the third base alone are (is) supplied by the *J_K* segment and the rest of the bases by the germ-line *V_K* segments^{21,23,73}. Furthermore, at least one κ chain case is known where the 96th residue is deleted entirely, and another case was found where an amino acid encoded by the 96th triplet of the *V_K*

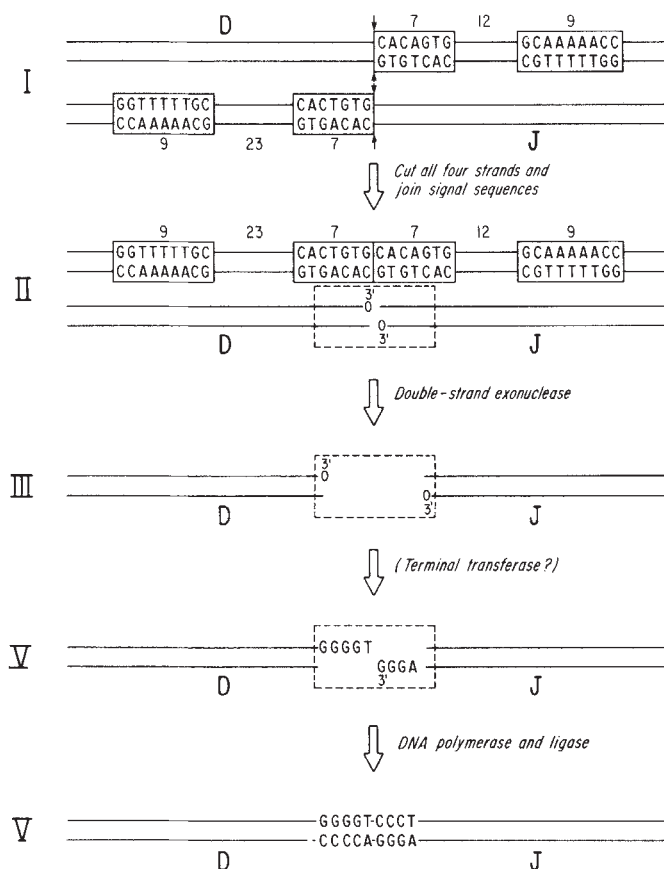


Fig. 5 Alt-Baltimore model for D - J joining⁷⁸. At stage I, the two joining gene segments are lined up for joining with the help of the DNA-binding proteins. All four DNA strands are nicked exactly at those ends of the signal heptamers adjacent to the coding sequences (indicated by arrows). At stage II, the two heptamers are joined intact in a tail-to-tail fashion. The coding sequences do not join but are held by protein so that their proximity is retained. At stage III, some nucleotides may be removed from these ends by an exonuclease(s). At stage IV, one or more nucleotides may be added at these ends in a template-free fashion. This may be carried out by terminal deoxynucleotidyl transferase which is found in bone marrow as well as in thymus and polymerizes random deoxynucleotides at 3' ends. To finish the joining process (stage V), a DNA polymerase replicates the added bases and a ligase seals the structure. It has been noticed that when no bases are added between D and J_H , there is an exact overlap of one or more bases from D and J_H at the joint⁵³. This may be explained if we assume that replicative polymerization of nucleotides begins from the hybrid formed transiently by the overlapping sequences before terminal transferase comes into action. The apparent lack of insertion at the join of light-chain genes may be explained by the fact that V_L - J_L joining occurs later in B-cell development than V_H - D - J_H joining when terminal transferase is absent from the cells.

segment appears as an insertion⁷³. These facts suggest strongly that the joining ends of the V_k and J_k segments can vary from one recombination event to another by several nucleotides²². In the case of J_H segments, this variability seems to extend over as many as 10 nucleotides²².

A striking case for the imprecise joining end was demonstrated by analyses of two joined D - J segments isolated from a myeloma QUPC52 and a cytolytic T-cell clone, B6. Both joinings occurred between D_{Q52} and J_{H2} and yet both the 3' joining end of D_{Q52} and the 5' joining end of J_{H2} are different in the two recombinations⁵².

Reminiscent of the imprecise joining end of a D segment is the fact that the D segment of an assembled heavy-chain gene is frequently several nucleotides shorter than the apparently

corresponding (except for one or a few base substitutions which are probably attributed to somatic mutations as discussed below) germ-line D segments at one or both ends (Fig. 3)⁵³. It is almost certain that this type of sequence discrepancy between the germ-line and somatic D segments arises from joining imprecision.

Because all immunoglobulin gene segments seem to be translated in only one reading frame in order to give rise to a functional immunoglobulin chain, one implication of this imprecision is that these gene segments often join in an out-of-phase reading frame. This type of non-productive joining occurs frequently in lymphatic cells^{53,73-77}. Thus, diversity is achieved at the expense of some waste.

Insertion at the joins

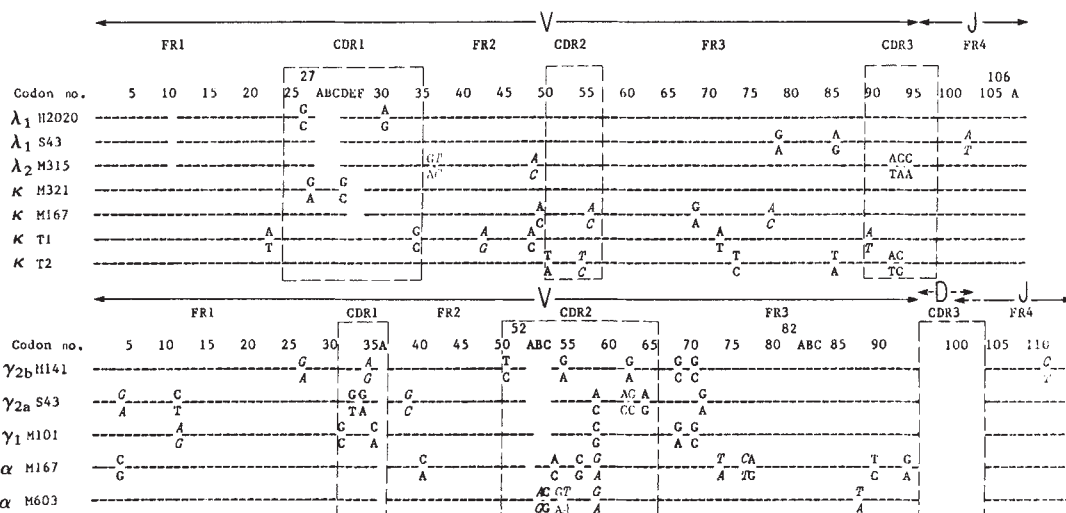
Two lines of observation suggest strongly that one or more nucleotides are inserted at the join of D and J_H segments and also probably at the join of V_H and D segments. In myelomas, hybridomas and other B and T lineage lymphocytes, joined D - J segments thought to be intermediates of V - D - J joining have been found on the allelic chromosome 12^{51-53,78,79}. In five of the eight D - J segments studied by DNA sequencing, one to four base pairs of unknown origins appear at the junction of the D and J_H segment⁵³. One possible origin of these extra base pairs is a second germ-line D segment^{52,53}. If this interpretation is correct, we must assume that two D segments can join. However, an extensive search for a joined D - D segment has been unsuccessful (C. Wood and S.T., unpublished). Although these negative data do not eliminate the possibility of D - D joining, they do increase the attractiveness of an alternative interpretation, that nucleotides are inserted without a template^{53,78}.

This possibility has also been suggested by comparisons of the germ-line and somatic D sequences, which show that extra nucleotides are added at the joins of both V_H - D and D - J_H segments⁵³. As illustrated in Fig. 3, one to several nucleotides at both ends of many somatic D sequences cannot be explained by the apparently corresponding germ-line D segments. Interestingly, these nucleotides are often a run of Gs or Cs⁵³. A model of V - J and V - D - J joining which contains an explanation for the origin of these nucleotides has been proposed⁷⁸ and is described below.

Mechanism of V - J and V - D - J joining

Earlier studies using rabbit allotypic markers showed that the V and C regions of a single chain are almost always encoded in the corresponding DNA segments residing in *cis* configuration^{80,116,117}. This suggested that the V - J and V - D - J joinings are intrachromosomal recombinations. *A priori*, an intrachromosomal recombination can occur according to any one of a combination of several different basic models. A specific gene segment may be duplicated and the copy inserted at a site adjacent to the second gene segment (copy-insertion)⁸¹. Alternatively, a specific gene segment may be excised into an episome-like structure and this in turn is integrated adjacent to the second gene segment (excision-insertion)⁸¹. The third possibility is that DNA in the interval between the two joining gene segments loops out, is excised, and is diluted out on subsequent cell multiplication (looping out-deletion)⁸¹. A fourth possibility is that the two gene segments to be joined are in the opposite orientation in the germ-line genome and the DNA between a particular gene segment (inclusive) and the second gene segment (exclusive) is inverted (inversion)⁸¹. Yet another possibility is that during the mitotic duplication of a chromosome an unequal crossing-over occurs between the 3' end of a gene segment on one sister chromatid and the 5' end of the second gene segment on the second sister chromatid (sister chromatid exchange)^{82,83}. The first Southern experiment addressing this question, in which the fate of the sequence

Fig. 6 Those mutations that have been documented by comparing the nucleotide sequences of a rearranged immunoglobulin gene and its corresponding germ-line gene segments are compiled. Where the corresponding nucleotides are identical, they are indicated by dashes; where different, the germ-line and somatic nucleotides on the coding strands are shown above and below, respectively. Neutral changes are indicated in italics. Data for the heavy-chain CDR3 are not given because of the uncertainty in the correspondence of a germ-line *D* segment to the somatic *D* region in question. References are: H2020A1 (ref. 19), S43A1 (ref. 109) and M315A2 (ref. 109), M321 κ (O. Bernard, G. Heinrich and S.T., unpublished), M167 κ (refs 96, 97), T1 and T2 κ (ref. 110), M141 γ 2b (ref. 22), S43 γ 2a (ref. 105), M101 γ 1 (ref. 114), M167 α and M603 α (ref. 24). All rearrangements but T2 κ are normal. T2 κ is rearranged in a wrong reading frame. Numbering of codons is according to the system used by Kabat *et al.*³⁵ which includes alphabetical letters A, B, C, etc. to denote some codon positions in order to maximize the sequence homology among the V regions of various species.



immediately 5' to the $J_{\lambda 1}$ segment and immediately 3' to the $V_{\lambda 1}$ segment was examined, gave a result in favour of the looping out-deletion model²¹. A subsequent experiment demonstrating the absence of a 5'-flanking region of the J_{κ} cluster and a 3'-flanking region of a V_{κ} segment in some κ -producing myelomas confirmed the deletion model⁸⁴.

However, Zachau and his co-workers found that some myelomas retain a J_{κ} heptamer joined with a V_{κ} heptamer in tail-to-tail configuration^{82,85}. In spite of the apparently reciprocal structural feature of this type of recombinant and the V_{κ} - J_{κ} recombinant, the joined signal sequences were not the reciprocal product of the recombination that brought together the V_{κ} and J_{κ} segments expressed in the myeloma^{82,85}. These results suggested strongly that in the κ locus of some myelomas more than one recombination has occurred and that looping out-deletion cannot be the only mechanism. In agreement with these results, Van Ness *et al.* found that a 5'-flanking sequence of the J_{κ} cluster remains in a new sequence context in some myelomas and in some κ -bearing B cells⁸³. On the basis of these results, Höchtel *et al.* and Van Ness *et al.* suggested the involvement of sister chromatid exchange in V_{κ} - J_{κ} joining^{82,83}.

However, the combination of inversion and deletion can also explain all existing findings⁸⁶ and a recent finding of a truly reciprocal product in κ myelomas⁸⁷ favours the involvement of inversion. Note that except for one *D* segment (D_{Q52}) and J_H segments, we remain ignorant about the relative orientation of various clusters of gene segments in the germ-line genome. A sister chromatid exchange between two joining gene segments of an opposite orientation will cause the loss of chromosomes on which these gene segments lie. Another testable criterion is that a segment of chromosome may be duplicated if a sister chromatid exchange occurs⁸⁶.

Several findings, all mentioned above, are relevant in considering the V - J and V - D - J joining mechanism at the fine structure level. First, the apparent by-product of V_{κ} - J_{κ} joining is composed of the heptamer 5' to a J_{κ} joined directly with the heptamer 3' to a V_{κ} in a tail-to-tail fashion. Second, the coding end of a given gene segment may differ by several nucleotides between two separate joining events. Third, in the case of heavy-chain genes, one to several nucleotides of unknown origin are apparently inserted at the join during the joining event. Based on these findings, Alt and Baltimore have proposed⁷⁸ the model outlined in Fig. 5. The details of the model are described in the legend.

Somatic mutation

The idea that mutation is a somatic diversifier of antibodies goes as far back as the middle of the 1950s^{88,89}. During the subsequent two decades, there have been extensive debates about this hypothesis and the alternative germ-line hypothesis (that is, for each immunoglobulin chain sequence there is a germ-line gene encoding it)^{3,4,46,90-94}. The first cogent evidence for the mutation theory was obtained by comparing the diversity of mouse $\lambda 1$ chains and their genes. Cohn, Weigert and their co-workers determined the V_{λ} region sequences of 19 different BALB/c and NZB $\lambda 1$ myelomas and found 12 of them to be identical (prototype sequences) and the other 7 different not only from each other but also from the prototype sequence by one to three residues (variant sequences)^{25,42,95}. Brack *et al.*¹⁷ and Bernard *et al.*¹⁹ showed that the mouse has a single $V_{\lambda 1}$ segment per haploid genome and that its nucleotide sequence corresponds exactly to that of the prototype $V_{\lambda 1}$ region: the variant sequences must therefore have arisen by somatic mutations.

Several subsets of κ and heavy chains and their germ-line V segments have been analysed by cloning and sequencing. These results all confirmed that somatic mutations amplify the diversity encoded in the germ-line genome (Fig. 6). Some examples are briefly described below. The V_{κ} probe prepared from the κ gene expressed in MOPC167 typically detects only one major band in the Southern blot analysis of total cellular DNA, allowing a relatively easy sequence comparison of the germ-line V_{κ} segment and its rearranged counterparts from two myelomas (MOPC167 and MOPC511)^{96,97}. Four and five mutations were found in the MOPC167 V_{κ} and in the MOPC511 V_{κ} , respectively; none of the mutations in the two expressed genes were identical.

Recently, a set of V_{κ} regions referred to as the V_{κ} -21 group, which show a more typical κ diversity pattern, has been analysed. A large number (>30) of κ chains of this group were identified, sequenced and classified into seven different subgroups^{98,99}, where a subgroup is defined as a set of V regions sharing subgroup-associated residues¹⁰⁰. Within a subgroup, the sequence variability of the V_{κ} regions is very similar to that of entire $V_{\lambda 1}$ regions: they consist of both prototype and variant sequences (see above). An earlier saturation hybridization experiment¹⁰¹ and the recent sequencing analysis of the germ-line V_{κ} -21 DNA segments (G. Heinrich and S.T., unpublished) strongly suggested that each subgroup is defined by a single

germ-line V_{κ} segment coding for the prototype V region and that the variant V regions arose by somatic mutation.

Similar observations have been made in heavy chains and, in particular, those V_H regions associated with the binding of phosphorylcholine (PC). Of the 19 V_H regions of anti-PC antibodies for which complete or nearly complete amino acid sequences are available, 10 (five μ chains and four α chains) use a single sequence, denoted T15 (prototype sequence). The remaining nine (five γ chains and four α chains) of these V_H regions differ from each other and from the prototype sequence by one to eight residues (variant sequences)¹⁰². A cDNA probe containing the prototype V_H sequence detects four different germ-line V_H segments, one of which corresponds exactly to the prototype amino acid sequence. An analysis of sequence homology¹⁰³ and, in some cases, of the restriction maps in the 5' upstream regions¹⁰⁴, allowed the conclusion that the same germ-line gene also gave rise to all nine variant sequences.

In the case of anti-NP^b [(4-hydroxy-3 nitrophenyl) acetyl] antibodies¹⁰⁵, it was shown by comparison of the 5' upstream regions that a germ-line V_H segment should have generated a somatic V_H segment γ chain that differed by 10 bases.

As shown in Fig. 6, somatic mutations are not restricted to the V segments but have been shown to occur in J segments and D segments. I have tacitly assumed that the base substitutions observed between the germ-line and rearranged gene segments arise by mutations. The fact that these somatic changes occur by single base substitutions in the overwhelming majority of cases (63 out of a total of 67 changes) supports this assumption rather than either of the two alternative mechanisms previously proposed: reciprocal recombinations between two homologous V 'genes'^{92,93} and gene conversion (refs 106–108 and M. Geftter and M. Fox, personal communication). Both of these mechanisms require two or more copies of homologous V-gene segments. Because the evidence for one germ-line $V_{\lambda 1}$ segment is good, neither of these mechanisms can apply to this gene system. (Note that the germ-line $V_{\lambda 2}$ segment¹⁴ cannot supply any of the bases required for the substitutions.) In the other gene systems hitherto studied, it is more difficult to eliminate these mechanisms because not all cross-hybridizing germ-line V-gene segments have been characterized by sequencing. Nevertheless, the studies on the more closely related set of germ-line V segments hitherto carried out have provided no supporting evidence^{103,109}.

Do lymphocytes have a hypermutation system which will alter bases specifically in the V genes^{89,90}, or are these base changes merely a consequence of an ordinary level of mutation (spontaneous mutation) followed by selection acting on the protein^{3,4}. The multiplication of B cells is stimulated by antigen, and those B cells bearing some mutant antibodies that fit better with an antigen may preferentially multiply. An alternative means of preferential B-cell stimulation is the idiotype-antiidiotype interaction¹¹⁸. Two observations, however, strongly suggest that the former hypothesis is correct. First, the frequency of mutation seems to be very high (Fig. 6). Second, the regions in which mutations are introduced are highly restricted to the V-J or V-D-J segments and, more significantly, to a few hundred base-pair untranslated regions surrounding the V-J or V-D-J segment. Because mutations in a particular untranslated region cannot be selected by a mechanism acting on the protein product, the above findings argue strongly against the hypothesis that these mutations arose by random spontaneous changes followed by selection operating on the gene products.

The existence of a hypermutation mechanism is not, of course, incompatible with the selection of the mutants by a mechanism acting on the protein. In fact, the density of mutations is about three times greater in CDRs than in FRs (Fig. 6). This could be explained without assuming selection if the putative hypermutation apparatus has a built-in mechanism to generate more mutations in CDRs. However, an alternative and more likely explanation is that those mutants with changes in

CDRs are selected at the protein level because of the primary role of CDRs in the interaction with antigens.

In some immunoglobulin genes, there exists a curiously high degree of preference for a purine on the coding strand to be mutated (ref. 119 and M. Weigert, personal communication). Thus, in the translated regions of two $\lambda 1$ chain (H2020 and S43), three κ chain (MOPC167, MOPC321 and T1) and three heavy-chain genes (M141, S43 and M101), 36 out of a total of 41 mutations occurred at purine residues in the coding strand. This may be considered as additional evidence for the existence of a hypermutational mechanism. However, the same tendency does not apply to the other four genes studied to date (M315 λ 2, T2 κ , M167 α and M603 α), where only 13 out of 30 mutations are purines.

As immunoglobulin gene sequences undergo two major somatic changes during B-cell differentiation—V-J or V-D-J joining and mutation—a question arises as to the temporal order of these events. Because there are far fewer mutations in the V_H regions of μ chains which are expressed first than in those of γ chains which are expressed later, Gearhart *et al.*¹⁰² and Bothwell *et al.*¹⁰⁵ concluded that V-D-J joining precedes mutation. They further suggested that somatic mutations may be linked in time and even in mechanism to switch recombination. If this hypothesis is correct, it may mean that all μ chains expressed on the surface of virgin B cells are products of rearranged germ-line V_H gene segments and that the mutation mechanism is called into action only after these B cells meet antigens.

Three considerations call this hypothesis into question, however. First, μ chains with mutated V_H regions have been found (C. Berek, personal communication). Second, the idea that somatic mutation is linked in mechanism to switch recombination is refuted by the finding of α chains with V_H regions encoded by germ-line sequences¹⁰². Third, and perhaps most important, we know very little about the extent of selection by antigen or other means (that is, anti-idiotype) and its effect on the relative occurrence of germ-line and somatically mutated V_H gene segments in the populations of various isotypes. It is possible, for instance, that mutation takes place during V-D-J joining. If the recombination accompanies mutations relatively infrequently and there is not much selection for mutants among the population of IgM-bearing cells, then most of the μ chains would carry germ-line-encoded V_H regions. Subsequent selection may drastically increase the fraction of mutants among IgG or IgA populations.

In three cases studied to date, unrearranged V_{κ} segments isolated from three myelomas have been shown not to carry mutations (ref. 96 and G. Heinrich and S.T., unpublished). In contrast, mutations are common among rearranged V_{κ} segments. At first glance, these results may suggest that mutations occur during or after V-J joining, but this interpretation is subject to the same criticism raised above concerning selection. Interestingly, in one of two cases where a V_{κ} segment has rearranged to a J κ segment in an out-of-phase reading frame, six mutations were found in the rearranged V_{κ} segment¹¹⁰. This V_{κ} segment does not produce a complete κ chain and is therefore presumably unselected. Analysis of more V_{κ} segments of this sort in comparison with unrearranged V_{κ} segments in myelomas may be very useful to our understanding of the possible relationship between V-J joining and mutation.

Virtually nothing is known about the molecular mechanism of the putative hypermutation. *A priori* mutations can be introduced either during semiconservative replication or repair synthesis. Because of the relative ease in accounting for the occurrence of these mutations in restricted areas of DNA, models presented to date generally favour the latter possibility. Brenner and Milstein suggested that a specific sequence near the V-C junction and in the C segment may act as a substrate for a DNA-nicking enzyme (note that it was assumed that V and C sequences are contiguous)⁹⁰. The nick is converted to a gap by a 3' exonuclease which is subsequently repaired by an error-prone polymerase and sealed by a ligase. In the light of new

information, this model obviously must be modified. One specific modification suggested by Selsing and Storb is that mutations are tied in mechanism to V - J (and perhaps to V - D - J) joining⁹⁶.

Conclusion: four somatic diversifiers

It is clear from the above that the principal mechanisms that diversify the germ-line-encoded genetic information for immunoglobulin chains have now been identified. The somatically generated diversity derives from four sources. The first type of diversity may be referred to as combinatorial diversity. This diversity arises from the fact that V_L and V_H regions are encoded by two and three different gene segments, respectively, each of which exists in multiple copies of different sequences, and that the joining can occur in various combinations in the population of lymphocytes. Thus, if the genome carries 2 V_L , 3 J_L , 300 V_H and 4 J_H segments, the maximum number of different V_L regions that could be somatically generated by combination is 1,206 (2×3 plus 300×4). Likewise, if there exist 200 V_H , 12 D and 4 J_H segments, the maximum number of different V_H regions would be 9.6×10^3 ($200 \times 12 \times 4$). In

reality, some of these combinations may not occur or occur very rarely (for example, $V_{\lambda 1}J_{\lambda 2}$).

Both the second and the third types of diversity occur in the joint regions and are referred to as junctional site diversity and junctional insertion diversity. The junctional site diversity arises at the V_L - J_L , V_H - D and D - J_H junctions because the joining ends are imprecise. The junctional insertion diversity seems to occur only in the V_H - D and D - J_H junctions where one to several nucleotides are inserted apparently in a template-independent fashion. Superimposed on these three somatic diversifiers based on recombination is somatic mutation, which alters bases throughout the sequences coding for the variable region.

The existence of the somatic diversifiers has now been established. While it is certain that these mechanisms are important for the function of the immune system, we remain ignorant about the details of the molecular mechanisms and their control in B-cell development. We are now in a position to resolve these issues.

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Regime of the Filchner–Ronne ice shelves, Antarctica

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Seismic, radio-echo, TWERLE balloon and oceanographic data of the Filchner–Ronne ice shelves are analysed. A large thin area in the central Ronne Ice Shelf is found to differ from the morphology of the Ross and Filchner ice shelves. This area is partly filled by basal saline ice, which can be attributed to regional regelation caused by oceanic circulation.

FILCHNER and Ronne ice shelves (both named Filchner Ice Shelf in FRG) form the second largest ice shelf in Antarctica with an area of 473,760 km² compared with 525,840 km² of Ross Ice Shelf, but their total volume of 307,300 km³ exceeds that of the Ross (224,500 km³) (ref. 1). Although their northern regions are divided by Berkner Island they form a continuous mass of ice in the south. Despite their significance, difficulties of access have meant that they have received less attention from glaciologists than has the Ross Ice Shelf.

Difficulty in interpreting weak radio-echo returns from the central part of Ronne Ice Shelf and uncertainties of navigation during earlier survey flights have delayed compilation of ice thickness data obtained by radio-echo sounding. This article combines several studies of ice thickness together with other previously unpublished investigations to give a picture of ice thickness and flow regime and presents a model of ice and ocean dynamics which may help to explain the observed patterns.

Two types of data are presented. Figure 1 shows ice thickness, the height of the ice front, surface features visible on satellite imagery and the lines and points on which thickness data were collected. Figure 2 shows depths to the base of the ice shelf and depths to the sea bed from seismic soundings and along the ice front from marine echo sounding. Figure 2 also shows the relative strength of radio-echoes from the base of the ice shelf and our estimates of oceanic circulation beneath the ice. We discuss the accuracy, interpretation and relationship between these different factors.

Thickness measurements

Only radio-echo data have been used in drawing ice thickness isopleths in Fig. 1. Where moderate or strong echoes are shown on Fig. 2, thicknesses on Fig. 1 are unambiguous and should be accurate to 10 m. Where weak echoes only are shown, errors may rise to 30 m. The possibility that weak echoes may come from an internal horizon in the ice shelf rather than from the ice–water interface is discussed later, but is not considered likely.

Ice thicknesses at TWERLE (Tropical Wind, Energy conversion and Reference Level Experiment) data points come from radio altimeter measurements of surface elevation from balloons that drifted at the 150-mbar level over the Antarctic ice sheet². Over thin ice shelves, where a strong bottom echo was present, the recorded echo sometimes switched between top and bottom surfaces. This range jump gave ice thicknesses at 10 points (N. Levanon, personal communication).

Seismic shooting by the first two investigations listed in Table 1 did not normally show reflections from the base of the ice shelf but clear reflections from the sea bed were obtained³. Two satisfactory seismic measurements of ice thickness were made in 1965–66 near the Foundation Ice Stream⁴. Ice thicknesses on the 1957–58 traverse published by Behrendt³ came from barometric measurements of surface elevations converted to ice thickness by assuming hydrostatic equilibrium. These differed by up to 350 m from the radio-echo thicknesses and were discarded. Depths to the sea bed at seismic data points on Fig. 2 have been recalculated using our radio-echo thicknesses.

Navigation

Navigation on 1969–70 flights was based on solar navigation and terrestrial maps of limited accuracy. The location of these flight lines has now been revised using:

- (1) A map to 81° S compiled from Landsat imagery by the UK Directorate of Overseas Surveys (C. J. Lane) with an average accuracy of around 1 km near control points. Boundaries of ice rises are seen clearly and are located to within a few kilometres.
- (2) The US Geological Survey 1:250,000 Antarctic map series covering areas inland of the Filchner–Ronne ice shelves.
- (3) Ice thicknesses measured on later flights with more accurate navigation to which ice thicknesses from earlier flights can be fitted by small adjustments of flight tracks.

During 1969–70 solar navigation was impossible on the outer flight line parallel to the ice front across the centre of Ronne Ice Shelf. Consequently, there could be a lateral error up to 50 km on this line but this would not cause a significant change in the position of isopleths on Fig. 1. On most other flight lines and at TWERLE data points positioned by satellite interrogation, errors are now unlikely to exceed 10 km, which is adequate for discussion of these 'reconnaissance' maps.

Interpretation of weak echoes

Uncertainty as to whether or not weak echoes in the central Ronne Ice Shelf come from an internal reflecting horizon within the ice shelf or from the base of the floating ice have been largely resolved by Neal's detailed studies of echo strength variation over the Ross Ice Shelf⁵. He discussed four different situations:

- (1) Brine infiltration. Where brine has risen through a crevasse or chasm to the level of porous firn, it spreads horizontally into

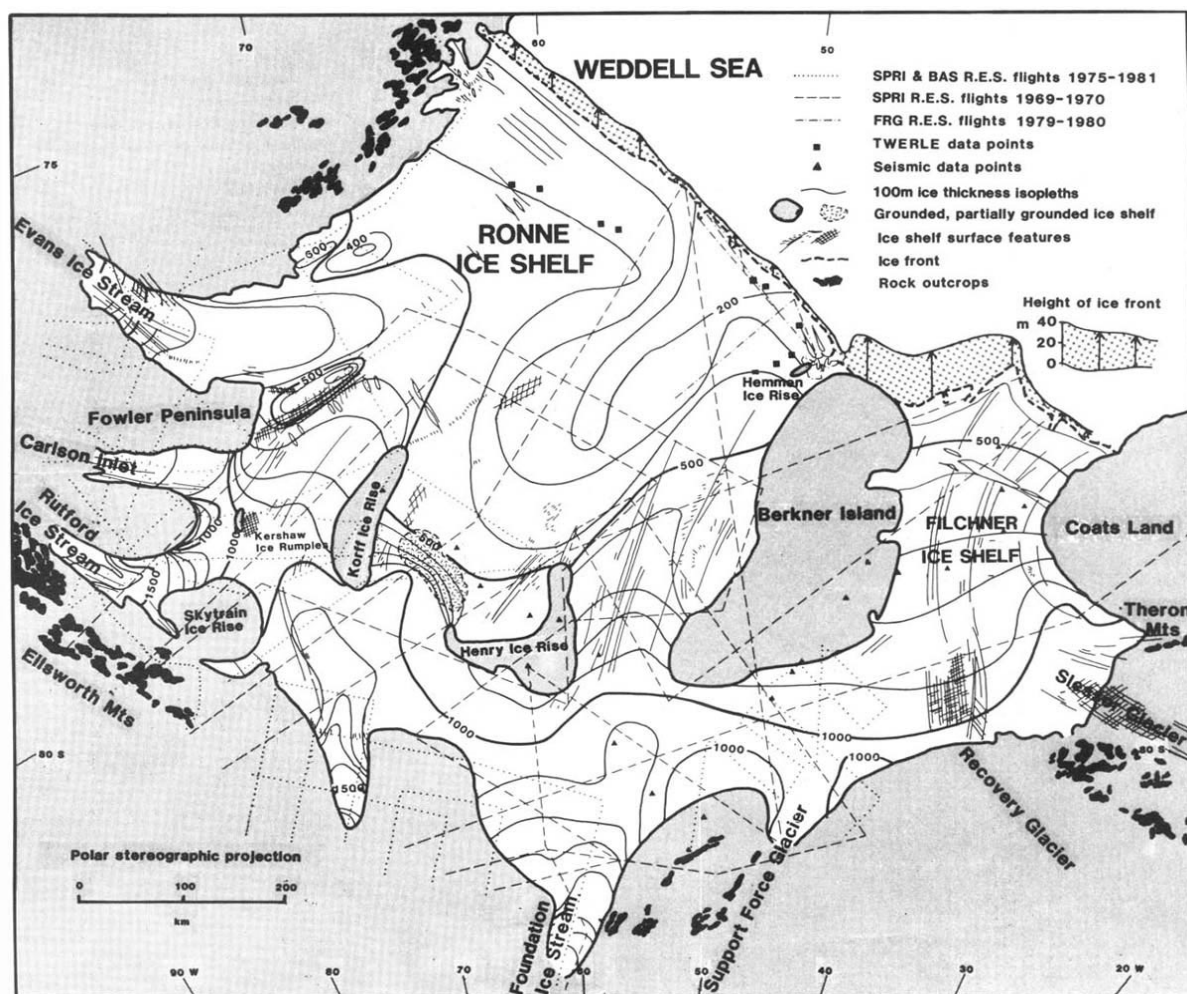


Fig. 1 Map of Filchner-Ronne ice shelves showing ice thickness isopleths, flight lines, seismic and TWERLE data points and flow features interpreted from Landsat pictures.

the firn⁶. This brine layer gives a strong reflection and occludes the bottom echo.

(2) Bottom crevassing. Upwelling of salt water into bottom crevasses, even after subsequent refreezing, is recognized by hyperbolic traces on radio-echo recordings. Crevassing can be due to tidal fracture near grounding lines or shear on the sides of fast moving ice streams entering a more slowly moving ice shelf. Relic crevasses are preserved and are carried down flow lines over long distances.

(3) Bottom freezing. Circulation of ocean water beneath an ice shelf of varying depth can cause either melting or freezing on to the base of the ice shelf due to changes in the freezing point of seawater with depth (pressure)^{7,8}. A saline ice layer due to bottom freezing reduces the bottom echo strength in two ways. The layer of saline ice absorbs radio echo energy at around $0.5\text{--}1.0\text{ dB m}^{-1}$ of two-way path length, compared with absorption around $0.02\text{--}0.04\text{ dB m}^{-1}$ in non-saline polar ice in the upper level of ice shelves. Furthermore, the echo return from the ice-seawater interface beneath such a basal ice layer shows a similar variation of intensity along the flightline to that from a rough bottom surface beneath inland ice. Neal calculates that the reflection coefficient from such a 'rough' ice-water interface can be up to 15 dB lower than that from a plane reflector. Neal also measured the reflection coefficient at an intermittent internal interface between average saline ice and 'polar' ice observed about 5 m above the basal echo on the Ross Ice Shelf to be -38 dB . The reflected energy from the ice-water interface at the same site was 28 dB below the incident energy at the top of the saline layer. The thickness of this saline layer varied with position but is similar to the 6 m of saline ice found⁹ sub-

sequently in the ice core at site J-9 on the Ross Ice Shelf some 365 km south-east of the site of Neal's observation.

(4) Where bottom melting occurs (and has removed any saline ice layer) strong echoes are found with a reflection coefficient approaching the theoretical maximum value of -1 dB for specular reflection from a smooth ice-seawater interface.

Observation on the Ronne Ice Shelf

The radio-echo system performance over the Filchner-Ronne Ice Shelf in 1969-70 (Table 1:4) is estimated at around 150 dB compared with 212 dB achieved during later NSF-SPRI-TUD studies including Neal's work on the Ross Ice Shelf and around 160 dB for the BAS and FRG studies (Table 1: 6, 9, 10). The long-range flights of the NSF-SPRI-TUD programme in 1978-79 (Table 1: 7, 8) did not cover the central Ronne Ice Shelf, so no equipment with the necessary system performance to record internal reflections from the top of any continuous layer of saline ice has been flown over the area where weak echoes are dominant. The system performance should have sufficed to record any strong echoes typical of a brine layer but these have not been seen although heavy crevassing has been noted, especially near the north end of Henry Ice Rise. In this area with ice thicknesses around 600 m, seawater would not rise to the level of the porous firn in a rift or chasm in the ice shelf.

The continuity of ice thickness as one moves from areas with strong echoes to areas with weak echoes suggests that we are following a continuous bottom surface, as no evidence of an internal reflecting horizon above the basal reflection has been found, as on the Ross Ice Shelf.

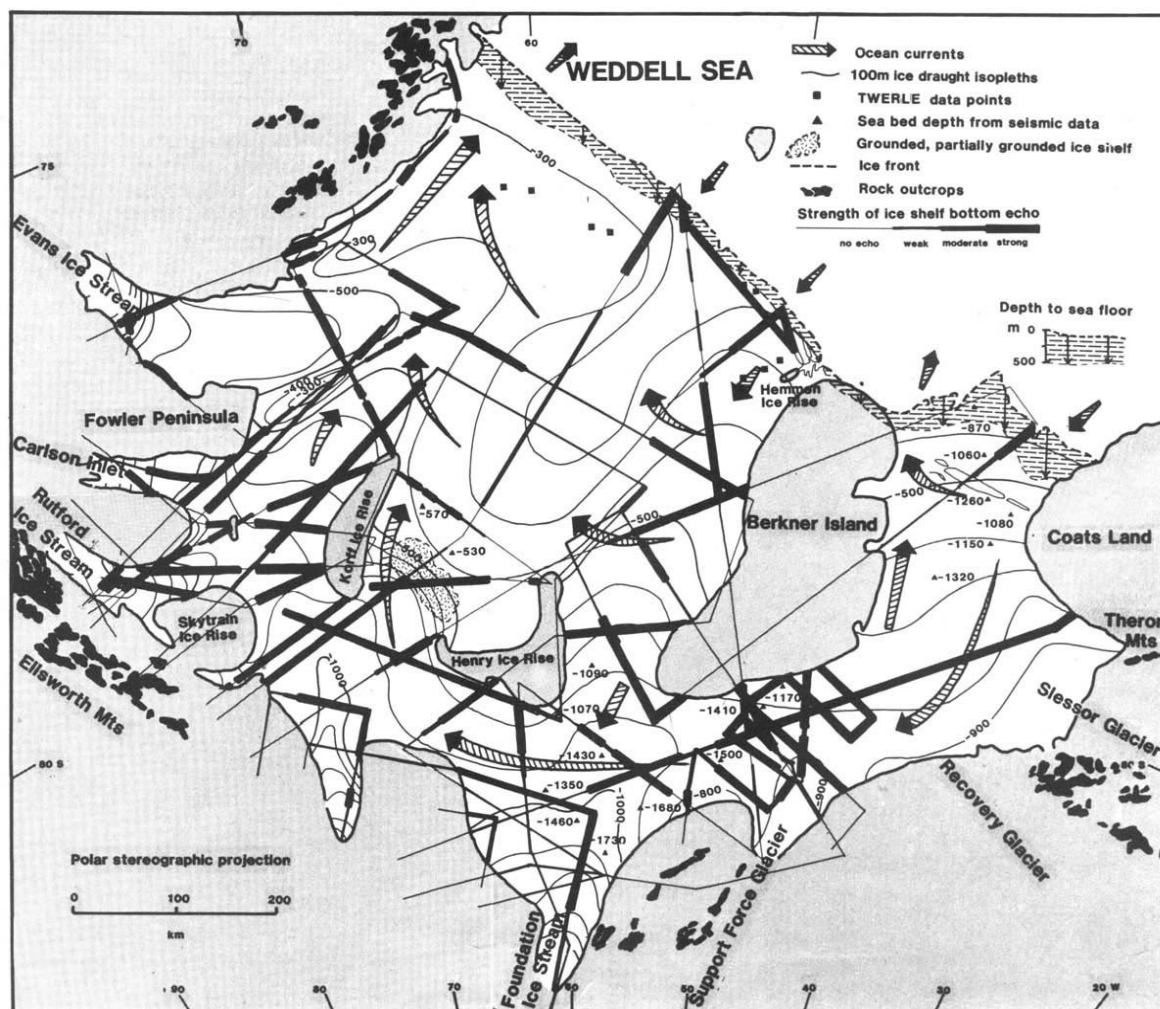


Fig. 2 Map of Filchner-Ronne ice shelves showing ice draught isopleths, depth to sea bed from seismic and oceanographic soundings, strength of radio-echo signal from the bottom of the ice and observed and inferred ocean currents.

An independent check on the presence of thinner ice over the central Ronne Ice Shelf comes from surface elevations measured by TWERLE balloons (N. Levanon, personal communication), even though the derived ice thicknesses may be up to 270 m in error. Mean ice thicknesses derived from all Levanon's elevation measurements between successive thickness isopleths in the central Ronne Ice Shelf shown in Fig. 1 are given in Table 2. From the standard error of the mean, it appears that there is satisfactory agreement between the two types of data to the 400 m isopleth. For greater thicknesses, Levanon's data indicate thinner ice, but this trend is the opposite to that which would be given by mistaking an internal reflection layer for the bottom reflection. Systematic errors appear likely, possibly becoming greater with distance from the ice front.

Ice dynamics

The flow and thickness of Filchner Ice Shelf appears to follow a similar pattern to that of Ross Ice Shelf^{10,11} and of Amery Ice Shelf¹². Surface features seen on Landsat images show that the principal sources of ice are from Slessor and Recovery Glaciers and from an unnamed ice stream to the north of the Theron Mountains; Support Force Glacier appears to be a less important source¹³. The ice streams converge between Berkner Island and Coats Land and at the ice front the velocity is highest near the centre at Belgrano and Ellsworth stations. Table 3 shows satisfactory measurements of the velocity of movement of the ice shelf which should give results within $\pm 10\%$. The only data inland of the ice front are those of Stephenson and Doake¹⁴ across Rutford Ice Stream where it is around 1,700 m thick and the ice is moving at $\sim 400 \text{ m yr}^{-1}$.

Estimates of movement obtained by comparison of ice front position from a marine survey in 1957 and an airborne one in 1966 with data from Landsat satellites in 1973 gave values of $1,000\text{--}2,000 \text{ m yr}^{-1}$ (refs 15, 16). Doppler satellite controlled marine surveys of the ice front in 1980 and 1981 (FRG expedition) yields an average advance of 1.8 km yr^{-1} for the Filchner Ice Front between 37° and 42° W , when compared with survey measurements made in 1957. Estimates based on mass balance and/or ice dynamics¹⁷⁻¹⁹ give velocities from 500 to $2,000 \text{ m yr}^{-1}$. All those estimates are subject to considerable errors due to the methods involved, but are nevertheless similar, or slightly too high, compared with those in Table 3.

On Ronne Ice Shelf the pattern of ice thickness (Fig. 1) and of surface features¹³ indicate that the major ice discharge occurs on the eastern and western sides of the ice shelf. Between Korff and Henry ice rises the ice thins rapidly by 300 m to 500 m in a distance of $\sim 15\text{--}50 \text{ km}$ over ice rumpled seen in Landsat pictures¹³. The relative absence of crevasses suggests that ice movement in this region is probably only tens of m yr^{-1} rather than the several hundred m yr^{-1} on the major ice streams to the east and west. The spread of thickness contours on the east side of Korff Ice Rise suggests appreciable but limited flow, in contrast to the western end of Henry Ice Rise where closely spaced contours suggest that flow is almost stopped there. A line of crevassing to the north-east of Korff Ice Rise indicates that movement is greater in the area to the north and west, which is fed from Rutford and adjacent ice streams^{13,31}.

The most striking feature of Ronne Ice Shelf is the zone of ice $< 200 \text{ m}$ thick stretching nearly 300 km in from the ice front. Bottom melting and the dynamics of ice flow can be considered

Table 1 Studies of thickness of Filchner-Ronne ice shelves

No.	Date of fieldwork	Description	Navigation method and probable errors (km)	Organization	Investigator
1	1957-58	Vehicle traverse/seismic and barometer	Astronomical 4	USARP	Behrendt ³
2	1957	Seismic around base	Terrestrial 2	TAE	Pratt ²⁹
3	1965-66	Helicopter/seismic 2 stations		USARP	Behrendt ⁴
4	1970	LR airborne radio-echo	Astronomical most and Terrestrial 1 flight 20 50	NSF-SPRI	Robin ³⁰
5	1974	Radio-echo from TWERLE balloons	Satellite 10	US-Israel	Levanon See text
6	1975	MR airborne radio-echo	Inertial fitted to Landsat images 3	BAS	Swithinbank ³¹
7	1977-78	LR airborne radio-echo	Inertial 3	NSF-SPRI-TUD	Drewry ²⁰
8	1978-79	LR airborne radio-echo	Inertial 3	NSF-SPRI-TUD	Drewry ³²
9	1980	SR airborne radio-echo	Terrestrial 10	FRG	Kohnen ²⁵
10	1981	MR airborne radio-echo	Doppler 2	BAS	Doake

Abbreviations: BAS, British Antarctic Survey; LR, long range; MR, medium range; NSF, National Science Foundation; SPRI, Scott Polar Research Institute; SR, short range; TAE, Trans-Antarctic Expedition 1955-58; TUD, Technical University of Denmark; TWERLE, Tropical Wind, Energy Conversion and Reference Level Experiment; USARP, United States Antarctic Research Program.

Table 2 Mean ice thicknesses between successive thickness isopleths in the central Ronne Ice Shelf

<i>R/e</i> mapped isopleth interval (m)	No. of observed elevations by balloon (<i>N</i>)	Mean thickness derived from elevations (m)	s.d. (m)	s.e.m. (s.d./√ <i>N</i> - 1)
100-200	6	210	123	55
200-300	9	250	142	50
300-400	16	330	142	37
400-500	20	289	145	33
500-600	11	422	171	54

as possible causes for this thin ice. Bottom melting could be due to the core of warm water flowing under the ice shelf between 50 and 56° W which is discussed later and shown in Fig. 2. However, if the thin ice zone were the result of extensive bottom melting, we would observe strong radio-echo reflections over all of this zone, which is not the case. This leaves the dynamics of ice flow as the cause of the thin ice.

Ice shelves normally become thicker in from the ice front. Their overall thickness is increased by drag along the sides opposing the forward motion²¹. In the central Ronne Ice Shelf the opposite process is at work: the two thick and rapidly moving ice streams on either side will tend to pull the central region of the ice shelf forwards. The shear on the sides of the central part is in the opposite sense to normal and will make the ice thin. The strongest shear gradients occur off the northern end of Korff and Henry ice rises where shear changes from restraint to pull and this may cause the high rate of thinning in the southern part of the central region. A three-dimensional analysis of the problem, taking account of shear and longitudinal stresses and stress gradients is complex²² and has not been attempted. We can, however, estimate an upper limit to the amount of spreading of the thick ice streams into the thinner ice shelf if we neglect the effect of shear stress on the land side of the ice stream and use the analysis of Thomas²³ and Sanderson²¹ to calculate the divergence angle ψ of an ice stream spreading by lateral straining. This is given by

$$\tan \psi = \frac{\lambda \dot{\epsilon}}{u} \quad (1)$$

where λ is the half-width of the ice stream, $\dot{\epsilon}$ the lateral strain rate and u the forward velocity. The lateral strain rate of an unconfined ice shelf is given approximately by

$$\dot{\epsilon} = \left(\frac{[\rho]gh}{2B} \right)^n$$

Table 3 Observed velocity of surface of ice shelf near the ice front

Location	Velocity (m yr ⁻¹)	Method	Ref.
Shackleton Station	77°59' S 37°10' W 1,135	Astronomical	33
Belgrano Station	77°59' S 38°44' W 1,372	Astronomical	33
Ellsworth Station	77°39' S 41°02' W 1,500	Astronomical	33
Ronne (Filchner Station)	77°09' S 50°38' W 1,042	Doppler satellite	34
	77°00' S 50°08' W 1,078	Doppler satellite	34

where $[\rho]$ is a density parameter related to the double integral of the density over the thickness of the ice, h ; B and n are parameters in the flow law of ice when expressed as $\dot{\epsilon} = (\sigma/B)^n$ where σ is the stress; and g is the acceleration due to gravity. For the case of an ice stream of thickness h_1 in an ice shelf of thickness h_2 , we assume that the resultant lateral strain rate to be used in equation (1) is due to a difference in lateral stress proportional to $(h_1 - h_2)$.

Then the divergence angle is given by

$$\tan \psi = \frac{\lambda}{u} \left(\frac{[\rho]g}{2B} \right)^n (h_1 - h_2)^n \quad (2)$$

Using values of $\lambda = 100$ km, $u = 500$ m yr⁻¹, $[\rho] = 50$ kg m⁻³ (ref. 14), $g = 9.81$ m s⁻², $B = 1.4 \times 10^8$ Nm⁻² s^{1/3} (ref. 14), $h_1 = 400$ m and $h_2 = 200$ m gives $\psi = 15^\circ$. This is the order of magnitude of spreading of the western ice stream into the thin area. The eastern (Foundation) ice stream appears to spread less rapidly, possibly due to its narrower width [equation (2)].

Sub-ice oceanic circulation

Information on circulation of water beneath the ice shelf can be obtained in two ways: by the study of inflow and outflow of water along the ice front and by inference from the strength of radio-echo reflections.

Oceanographic observations carried out during the FRG expedition in the summer of 1979-80 (ref. 24), revealed a complicated picture. The main features are a (relatively) warm water mass (-1.5 °C) with low salinity (34.40-34.65‰) centred around 200 m depth between 50° and 56° W. Gammelsrød and Slotsvik²⁴ deduce from CTD measurements that there is a general influx of warm low-salinity water in this area. Tidal measurements at the ice front at 49° W revealed strong tidal currents having velocities up to 40 cm s⁻¹ perpendicular to the

ice front. Strong vertical mixing was observed in conjunction with the outflow. Inflowing water was 0.2°C warmer than water flowing away from the front. High melt rates at the front and under the ice shelf near the ice front can be expected as a consequence of the warm water influx. This is manifested by the low ice front between 49° and 56°W and also by the wedge-like shape of the ice shelf recorded by radio-echo sounding of the profile at 50°W perpendicular to the ice front. Bottom melting of 3.2 m yr^{-1} has been deduced from surface measurements made by the FRG expedition in 1980, 20 km south of the ice front to the west of Berkner Island²⁵. It appears that the net inflow of water beneath Ronne Ice Shelf between 49° and 56°W leads to a net outflow beneath the western part of Ronne Ice Shelf. A similar situation exists beneath Filchner Ice Shelf with an inflow of water on the eastern side and a net outflow on the western (Berkner Island) side^{26–28}. Arrows indicating water flow at a level beneath the ice shelves are shown in Fig. 2.

Because of the variation of freezing point with depth, we expect melting of the ice when the ocean circulation is such that the water in contact with the underside of the ice shelf is being forced downwards, while if water is rising, saline ice can be frozen to the bottom of the ice shelf^{7,8}. It is therefore possible to relate the radio-echo strength pattern in Fig. 2 to where melting or freezing at the base of the ice shelf might occur and then to infer the oceanic circulation.

Although lacking calibrated echo strength data for most of the Filchner–Ronne Ice Shelf, differences are so great that general trends can be readily identified by the qualitative results in Fig. 2. These show the presence of strong echoes on the eastern Filchner Ice Shelf and on the southern, eastern and western parts of Ronne Ice Shelf, while the thinner ice of the central area seaward of Korff and Henry ice rises produces weak echoes only. We conclude that in the central zone of Ronne Ice Shelf a considerable amount of saline ice is present in the lower levels of the ice shelf. Weak echoes persist almost to the ice front between 52° and 54°W , despite strong melting near the ice front which finally removes the saline basal ice when the ice thickness is reduced to around 150 m.

The strong echoes on southern parts of the Filchner–Ronne Ice Shelf suggest that, in addition to the cyclonic circulation beneath the northern part of the Filchner Ice Shelf, a component of flow continues around the southern end of Berkner Island.

Beneath Ronne Ice Shelf, the warm current flowing in between 49° and 56°W appears to cause strong bottom melting along the Foundation Ice Stream outflow to the west of Berkner Island. Cyclonic circulation under this ice stream will produce saline ice as water is carried beneath the thinner central part of the ice shelf, and then as it moves farther across beneath the thicker western stream of this ice shelf, further melting will take place. Strong echoes on the western part suggest that cyclonic circulation beneath the seaward half of the Ronne Ice Shelf exerts a greater influence than that of possible circulation

moving around the Filchner Ice Shelf to between Korff and Skytrain ice rises. It is possible that some of this latter circulation may contribute to the freezing beneath the central Ronne Ice Shelf if some outflow takes place between Korff and Henry ice rises.

The pattern of almost continuous strong bottom echoes from the thick ice to the west and south of Korff Ice Rise shows that bottom melting occurs over the whole area without indicating any particular pattern of circulation. A relatively slow circulation would readily transport ample heat to cause considerable melting in this region, but the process by which this heat reaches the ice–water interface is not clear. Stephenson and Doake¹⁴ assuming steady-state conditions, estimate from their measurements of ice thickness, ice movement and surface strain that substantial bottom melting of around 1.8 m yr^{-1} takes place close to the grounding line of Rutford Ice Stream.

Heights along the ice fronts shown in Fig. 1 are generally consistent with the proposed pattern of strong melting beneath the eastern half of both Filchner and Ronne ice shelves, and with the greater height on their western sections. It must, however, be remembered that the height of an ice front depends also on the age of the ice front, as calving of vast ice bergs from these ice shelves will, at intervals, expose new ice cliffs some kilometres back from the previous ice front and where the ice is considerably thicker. The higher ice front between 40° and 45°W could also result from more recent calving than elsewhere.

Conclusions

A major feature of Ronne Ice Shelf is the thin ice in the central region. It occurs primarily because of the influence of grounded, or partially grounded, ice on and between Korff and Henry ice rises. Large ice streams draining the inland ice sheet are diverted along the eastern and western margins of Ronne Ice Shelf. Shear between these thick, fast moving ice streams and the slower moving ice which has flowed between Korff and Henry ice rises causes the central area to thin sufficiently for it to persist until the ice front. Cyclonic circulation of ocean currents raises water from beneath thicker ice to the thinner area, producing saline ice. Bottom melting occurs over most of the rest of Ronne Ice Shelf.

Filchner Ice Shelf has a more uniform thickness across the ice shelf. The deep trough in the sea bed seen at the ice front continues south of Berkner Island, where water more than 600 m thick lies beneath ice itself 1,000 m below sea level. Water appears to circulate from underneath the Filchner Ice Shelf to the Ronne Ice Shelf.

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Hinf family: a novel repeated DNA family of the human genome

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The isolation of a mutant adenovirus carrying an insertion of cellular DNA has led to the identification of a new family of human repetitive sequences, which are found tandemly arranged in the genome. The sequence of the viral insert resembles that of eukaryotic transposable elements.

MAMMALIAN genomes are characterized by families of repeated DNA sequences¹, although the functional significance of these sequences remains speculative. It has been proposed that some of the sequences are involved in regulation of expression of single copy sequences², or that these DNAs may be of the 'parasitic DNA' type^{3,4}. In the human genome, a family of interspersed repetitive DNA, the *Alu* family, of ~300 base pairs (bp), is present a minimum of 300,000 times in the haploid genome⁵. Many *Alu* family members have been shown to contain RNA polymerase III initiation sites^{6,7}, and it has recently been suggested that the *Alu* family members may be transposable elements, because direct repeats of 5–20 bp of non-*Alu* sequences flank *Alu* sequences^{8,9}.

A second family of repeated DNA is the long tandemly repeated sequences represented in satellite DNA^{10,11}. Cleavage of this repetitive DNA with restriction endonucleases yields DNA fragments which have lengths that are multiples of a monomeric sequence^{12–14}. It has been proposed that the basic subunit length of many satellite DNAs is ~170 bp¹⁵. One of the well characterized satellite sequences is the α -satellite DNA of African green monkey (AGM) which exists as long tandem repeats of a 172-bp monomer unit¹⁶ as well as in interspersed arrays¹⁷. An analogous family of repetitive sequences in human DNA which gives a series of multimeric fragments when digested with the restriction endonuclease *EcoRI* has been identified¹⁸. This sequence consists of two tandem dimerized units of 171 and 169 bp. A third family of repeated DNA contains short simple tandem repeats such as those present in the spacer region between ribosomal cistrons¹⁹ and class switch regions of immunoglobulin heavy chain gene²⁰. Boseley *et al.*¹⁹ have described tandem repeats of two related subunits occurring in the spacer region which therefore suggests a relationship between satellite and spacer evolution.

We describe here a human DNA sequence, designated the *Hinf* family, which occurs in tandem arrays at different locations in the genome, the repeats consisting of a 319-bp unit and occurring as a dimerized unit of two related but distinct subunits of 172 bp and 147 bp. The *Hinf* insertion present in an adenovirus 7 (Ad7) mutant resembles the structures of eukaryotic transposable elements^{21–23} and the integrated provirus of retrovirus^{24,25}.

Isolation of the Ad7 insertion mutant *in 721*

The DNA of Ad7 (strain Greider) has a single *EcoRI* cleavage site at map position (mp) 86.6 (ref. 26) which is situated at the 3' end of early gene block 3 (E3) located at mp 75 to 87.2 (ref. 27). Portions of E3 have been shown to be non-essential for growth of Ad2, Ad5 and Ad7 on cultured human cells^{28–30}. We have isolated several viable Ad7 mutants with deletions or

insertions within E3 (unpublished data). These mutants were isolated starting with the terminal protein–DNA complex which was prepared from a stock of Ad7 propagated at a moderately high multiplicity of infection (50 plaque-forming units per cell). The DNA–protein complex was digested with *EcoRI* and transfected into the Ad5 transformed human cell line, 293, with the aim of selecting mutants lacking the *EcoRI* cleavage site. The rationale for this approach is that the plaque-forming ability of wild-type DNA will be inactivated by cleavage with *EcoRI* but mutant DNA lacking the *EcoRI* cleavage site should yield plaques.

Of the 24 plaque isolates examined, 15 were deletion mutants lacking the *EcoRI* site. Surprisingly, four were insertion mutants retaining one or two *EcoRI* sites, despite the selective pressure against mutants with *EcoRI* cleavage sites. One of the insertion mutants (*in 721*), which has an insertion of ~460 bp and two *EcoRI* sites, was analysed in detail. *EcoRI* cleavage patterns of the DNAs of wild-type (wt) and *in 721* are shown in Fig. 1. Wild-type DNA yielded two *EcoRI* fragments, A (30,200 bp) and B (4,800 bp); however, mutant *in 721* DNA yielded three fragments, A and B similar in size to wt, and a new fragment, C, of ~460 bp. The *in 721* *EcoRI*-C fragment was sequenced and was used as a probe in Southern blot analysis with cellular and viral DNAs.

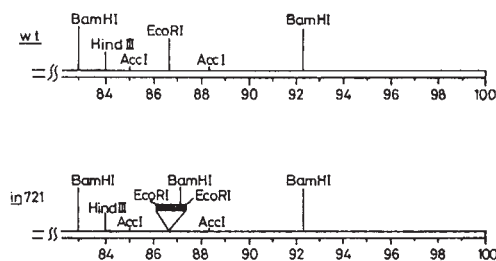


Fig. 1 *EcoRI* cleavage patterns and partial restriction maps of Ad7 and *in 721* DNAs. Mutant *in 721* was isolated essentially as described in the text. Ad7 DNA–terminal protein complex was prepared by the Sepharose–GuHCl method³⁹, digested extensively with *EcoRI* and transfected by the calcium phosphate method⁴⁰ on the 293 human cell line⁴¹. Viral plaques were picked and screened for mutants as described earlier⁴². Viral DNA was digested with *EcoRI* in standard conditions and fractionated on a 1.4% agarose slab gel, stained with ethidium bromide and photographed (left panel). Lane 1, *in 721* DNA; lane 2, wt DNA. Ad7 and Ad12 DNAs digested with *HindIII* were used as size markers. The partial restriction maps (mp 83–100) of Ad7 wt and *in 721* are shown in the right panel.

The insertion sequence is of cellular origin

To determine whether the 460-bp sequence inserted in *in* 721 is of cellular or viral origin, we performed Southern blot analysis on the DNAs of human cell line 293, Ad5 wt, Ad7 wt and *in* 721. The cellular and viral DNAs were partially or completely digested with various restriction endonucleases.

As shown in Fig. 2, the insertion sequence did not hybridize with Ad7 wt or Ad5 wt DNA fragments (lanes 6, 7) but did

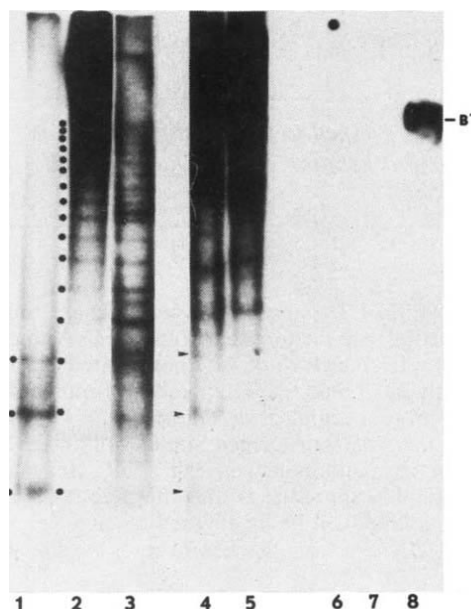


Fig. 2 Southern blot analysis of 293 human cellular and viral DNAs. DNAs from 293 cells (lanes 1–5) and from the various viruses (lane 6, Ad5 wt; lane 7, Ad7 wt; lane 8, Ad7 *in* 721) were cleaved with restriction endonucleases, fractionated on 1.4% agarose gels, transferred to nitrocellulose sheets¹⁴ and hybridized with ³²P-labelled ($1-2 \times 10^8$ c.p.m. per μ g)⁴³ *Hinf* insert (*Eco*RI-C fragment from *in* 721 DNA). Lanes 1 and 4–8 show complete digestion with the respective restriction endonucleases; lanes 2 and 3 show partial digestion with restriction endonucleases *Hinf*I or *Sau*3A. The size determinations were carried out using SV40 DNA digested with *Hinf*I and Ad7 DNA digested with *Hind*III as markers.

hybridize to the *Hind*III-B' fragment of *in* 721 (lane 8), which contains the insertion sequence, and to multiple fragments of 293 cellular DNA, indicating that the insertion sequence is of cellular origin.

When 293 cellular DNA was completely digested with the restriction endonuclease *Hinf*I the insertion sequence hybridized to three fragments of about 320 bp, 640 bp and 960 bp (Fig. 2, lane 1). On the other hand, when the cellular DNA was partially digested with *Hinf*I, multimers of 320 bp (indicated by the dots in lane 2) were hybridized, indicating that sequences homologous to the insert DNA are present as tandem repeats of a unit ~320 bp long. As this repeated DNA is cleaved into monomeric units with *Hinf*I, we have designated this DNA family the *Hinf* family. In lane 1, where the cellular DNA was completely digested with *Hinf*I, the appearance of the 640- and 960-bp fragments may be due to the loss of some of the *Hinf*I recognition sites during the evolution of this DNA family. As seen in lane 3, when cellular DNA was partially digested with *Sau*3A, bands of intermediate molecular weight were revealed in addition to the 320-bp multimeric units. These are probably due to new *Sau*3A sites (in addition to the sites located at 320-bp intervals) generated during divergence of this DNA family. As *Sau*3A has a 4-bp recognition sequence (5'-GATC-3'), there are 20 sites within the *Hinf* family where a single base pair alteration would generate a new site.

Restriction endonucleases *Eco*RI and *Hind*III do not have cleavage sites within the *Hinf* family, as revealed by our DNA sequence data (see below). However, 293 cellular DNA, when cleaved with *Hind*III and analysed by a Southern blot, revealed multiple bands of DNA homologous to the *Hinf* family (Fig. 2, lane 5). As multiple bands are generated with *Hind*III, we conclude that tandem repeats of the *Hinf* DNA occur at different locations on the cellular genome. The *Hinf* DNA sequence does not contain regions that could readily mutate to generate *Hind*III sites.

As seen in Fig. 2, lane 4, when the cellular DNA was cleaved with *Eco*RI, several DNA bands homologous to the *Hinf* family were observed. Some of these bands are in a size range of less than 1,500 bp (denoted by the arrows in lane 4). Careful analysis of the *Hinf*I-digested and *Eco*RI-digested DNA blots revealed that these DNA bands of lower size generated by *Eco*RI are distinctly larger (for example, 350, 700 and 1,100 bp) than the DNA fragments generated by *Hinf*I (for example, 320, 640, 960 bp; lane 1). We believe that at least some of the DNA bands denoted by the arrows may be due to dispersed partial or complete monomer units of the *Hinf* family flanked by *Eco*RI sites located in non-*Hinf* sequences. Alternatively, some of the bands may be generated by a single base pair change in a

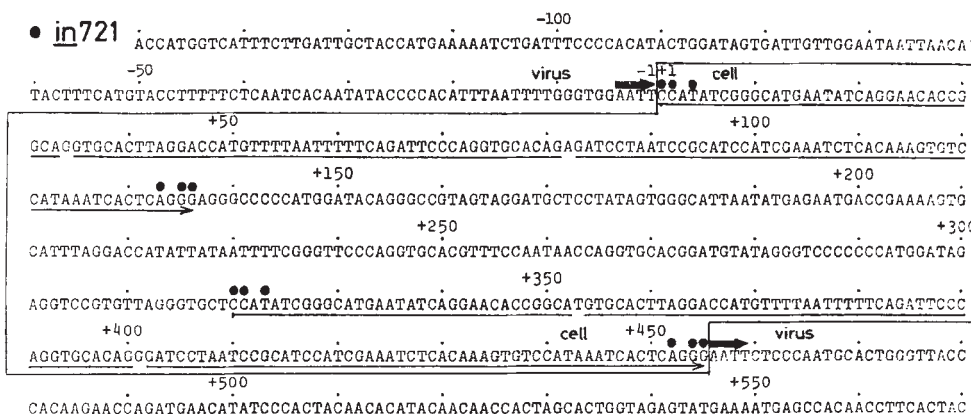
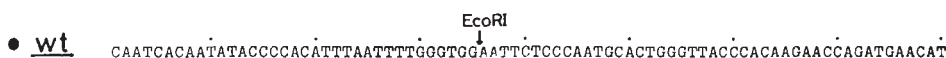


Fig. 3 Nucleotide sequence of the *Hinf* insert and the Ad7 genome at the insertion site. *Hinf* sequences are shown within the boxed area. Direct repeats are marked by solid thin arrows and inverted repeats are marked by filled circles. Duplicated viral sequences are marked by solid thick arrows.



I 65 GATTCCACAGG TGCACAGAGA TCC TAATC CGCATCCATC GAAATCTCAC AAGATGTCCA
 II 237 GGTTCACAGG TGCAC GTT TCCAATAA C CA GGTG CA

I 123 TAAATCACTC AGGAGAGGCC CCCATGGATA CAGGGCCGTA GTAGGATGCT CCTATAGTGG
 II 272 CGGATGTAT AGGG TCCCC CCCATGGATA GAGGTCCTGT TTAGGCTGCT CC ATATCGG

I 183 GCATTAATAT GAGAATGACC GAAAGTGCA TTTAGGACCA TATTATAATT T TCG
 II 329 GCATGAATAT CAGGAACACC GGCAATGTGCA CTAGGACCA TGTTTAATT TTCA

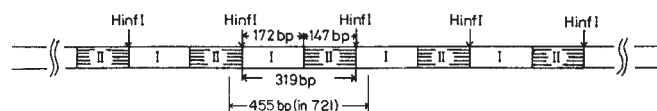


Fig. 4 Homology between *Hinf* family subunits I and II and organization of tandemly repeated *Hinf* family DNA. The organization (that is, tandem repeats of a 319-bp unit) is deduced from Southern blot and DNA sequence analysis and is shown at the bottom of the figure. The homology between the two subunits is shown in the upper panel. The sequence variations in subunit II are indicated by the dots.

monomer unit, yielding a new *EcoRI* site. There is a single site within the *Hinf* monomer at +103 to 108 or +422 to 427 in Fig. 3 where a single base pair change would generate an *EcoRI* site.

DNA sequence analysis of the *Hinf* element and the site of integration

(1) Viral DNA has a 4-bp duplication at the site of the *Hinf* element insertion: The nucleotide sequence of the *Hinf* insert, the viral sequences contiguous to the site of integration on the Ad7 mutant (*in* 721) genome, and the corresponding region of the wt genome, were determined by the method of Maxam and Gilbert³¹.

As shown in Fig. 3, the *Hinf* insert present in the *in* 721 genome is 455 bp long (shown within the boxed area). This insert is flanked on both sides by four bases 5'-AATT-3' (marked by solid arrows) of viral DNA at coordinates -4 to -1 and +456 to +460. The same 4-bp sequence is present only once at the corresponding site on the wt genome (at the single *EcoRI* site, mp 86.6). The four bases AATT duplicated in *in* 721 DNA also form the single-stranded protruding ends generated by restriction endonuclease *EcoRI*³². The duplication may therefore be a consequence of precleaving the viral DNA-terminal protein complex with *EcoRI* and the subsequent integration of the *Hinf* element (see below). The duplication of the four bases at the integration site in conjunction with nucleotide sequences present at the ends of the *Hinf* element generate two *EcoRI* cleavage sites which enables precise excision of the *Hinf* element from the genome of *in* 721 with *EcoRI*.

Although the DNA sequences presented in Fig. 2 are compatible with the possibility of 5 or 6 bp duplication (that is, 5'-GAATT-3' or 5'-GGAATT-3'), we believe that the duplication is only 4 bp, because the dinucleotide GG present at the 3' end of the 455-bp insert (that is, at positions +454 and 455) is part of the second *Hinf* direct repeat. Note the presence of these dinucleotides at the 3' end of the first direct repeat at positions +135 and +136.

(2) The inserted *Hinf* element has direct terminal and inverted terminal repeats: As seen from the DNA sequence data (Fig. 3), the inserted *Hinf* element has a direct terminal repeat of 136 bp (marked by thin arrows) between positions +1 to +136 and between +320 to +455. There are two single base pair mismatches between the two direct repeats. In the first mismatch there is a G to T transversion at positions +34 and +351 (that is, the first direct repeat has a G at +34 whereas the second direct repeat has a T at the corresponding position, +351). The second mismatch is at positions +82 and +410 where there is an A to G transition. The sequence data also

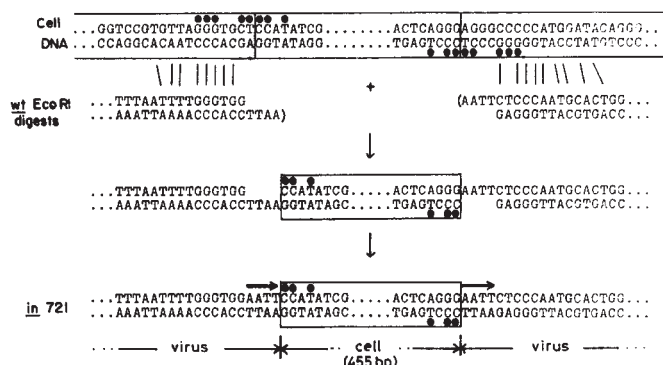


Fig. 5 Structural features near the site of insertion and the molecular consequence of *Hinf* element insertion. DNA sequence homologies between the cellular *Hinf* DNA (flanking the 455-bp insert) and Ad7 viral DNA are indicated by vertical lines. The inverted repeats of the *Hinf* family are shown by filled circles. The inserted *Hinf* DNA is shown within the boxed area. The solid arrows indicate the duplicated viral sequences.

reveal a short 2-bp (or 4-bp with 1-bp mismatch) inverted terminal repeat (5'-CC-T A-GG-3') marked by filled circles in Fig. 3.

Organization of the *Hinf* family in cellular DNA

The Southern blot analysis using partial digestion with *HinfI* or *Sau3A* indicated that *Hinf* family DNA exists mostly as tandem repeats of a unit of ~320 bp. In Fig. 2, lane 2, up to ~17 repeats of the 319-bp unit could be counted. This is a minimum estimate because the resolution near the top of the gel is insufficient to count the individual bands. As mentioned earlier, families of these tandem repeats seem to be located at different locations on the cellular genome (Fig. 2, lanes 4, 5). The total number of *Hinf* elements in the 293 genome has been estimated as 50–100 copies per haploid genome (data not shown) using a semi-quantitative Southern blot analysis³³.

According to the DNA sequence data, the tandemly repeated DNA could be divided into subunits of 319 bp bound by *HinfI* cleavage sites. The nucleotide sequence within the 319-bp *Hinf* monomer could be divided into two related but distinct subunits of 172 bp (subunit I) and 147 bp (subunit II), based on homology. Comparison of the two subunits located within the 455-bp *Hinf* element present in *in* 721 genome is shown in Fig. 4, and reveals ~70% homology between the two subunits. The striking difference between subunits I and II is the deletion of 20 bp in subunit II in addition to other smaller deletions and insertions (Fig. 4). The 455-bp insert present in *in* 721 genome represents 1.4 units (Fig. 4, bottom panel), thereby generating two terminal direct repeats of 136 bp each.

Sequence characteristics at the integration site

The *Hinf* sequences present on the cellular genome continuous with the 455-bp insert present in the *in* 721 genome can be extrapolated because the *Hinf* family sequence is tandemly repeated. Comparison of the cellular and viral DNAs reveals a patchy homology with the Ad7 genome at the integration site as illustrated in Fig. 5. There is a 8-bp homology on the left-hand side (with 2-bp mismatch) and a 9-bp homology on the right-hand side (with 4-bp mismatch). In addition, the cellular *Hinf* DNA has an imperfect inverted repeat of 8 out of 11 bp (marked by filled circles in Fig. 5). Three of the 11 bp (5'-CC-T A-GC-3') constitute the inverted terminal repeat present in the *Hinf* insert of *in* 721. These sequences may have some role in the integration of the *Hinf* element.

At the site of insertion, Ad7 wt is capable of forming a hairpin of 19 bases which includes the *EcoRI* cleavage site at which the *Hinf* element is inserted (Fig. 6). Unintegrated *Hinf* DNA as well as integrated *Hinf* DNA is capable of forming stable

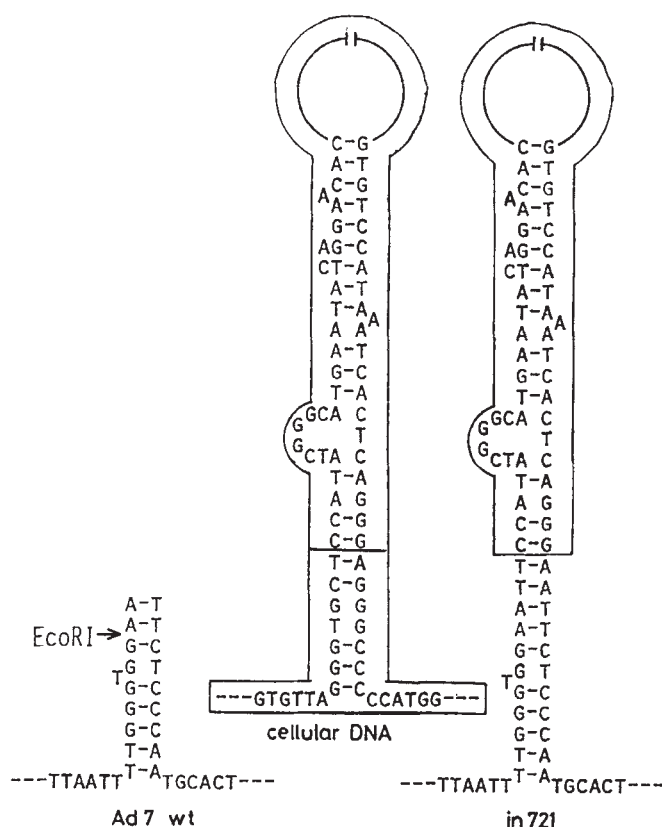


Fig. 6 Possible secondary structures at the integration site of Ad7 wt, *in* 721 and unintegrated *Hinf* DNA.

stem-loop structures as illustrated in Fig. 6. These structures may also be involved in the integration and excision of the *Hinf* element.

Discussion

We have identified a new human repeated DNA family designated the *Hinf* family by direct DNA sequence analysis of a 455-bp human cellular DNA sequence present in the genome of an Ad7 mutant (*in* 721). The basic structure of the *Hinf* family resembles a 340-bp tandemly repeated satellite-like DNA designated *EcoRI* multimers¹⁸. The 340-bp *EcoRI* monomer has been shown to consist of two related dimerized subunits of 169 and 171 bp. The human *Hinf* sequence we have

identified also reveals two related dimerized subunits of 172 and 147 bp. Such dimerized subunits have also been reported in a Bonnet monkey satellite sequence^{34,35} as well as in ribosomal spacer DNA¹⁹. Models based on unequal double-strand exchanges associated with DNA replication have been proposed for the creation and amplification of these DNA sequences^{12,13,19}. Comparison of the nucleotide sequence of *Hinf* family DNA with other human repeated DNAs revealed no significant homology.

Although we do not know the exact mode of *Hinf* DNA insertion into the viral genome, there are two likely possibilities. The first is a double nonhomologous recombination between the *Hinf* sequence and two restriction fragments (transfected into human cells during the isolation of viral mutants) of the Ad7 genome whose staggered *EcoRI* ends were first repaired by cellular DNA polymerases generating the 4-bp viral DNA duplication. The other possibility is recombination with a single target DNA molecule with double-strand breaks at the *EcoRI* site, using a mechanism proposed for integration of transposable elements^{36,37}. Such Ad7 genomes with double-strand breaks would be generated by the annealing of two *EcoRI* fragments transfected into the cells via their cohesive ends. We believe the latter possibility may be the likely mode for the *Hinf* insertion, resulting in the duplication of the target site (see Fig. 5). We have observed that Ad7 DNA-terminal protein complex digested extensively with *EcoRI* by several rounds of enzyme digestion always yielded 10–25% of Ad7 wt plaques which may be due to the annealing of the two viral DNA fragments and subsequent ligation inside the cells rather than the residual undigested DNA. A number of other insertion mutants bearing a duplication of the cohesive ends generated by restriction endonucleases have been isolated using a protocol similar to that used here (G.C., unpublished, and N. Jones, personal communication).

The integrated *Hinf* element has a terminal direct repeat of 136 bp and each direct repeat is bounded by a 2-bp inverted terminal repeat and these structures resemble several transposable elements described in eukaryotic cells^{21–23} and integrated retroviral genomes^{24,25}. Although the inverted terminal repeat of the *Hinf* insert is only 2 bp (or 4 bp with 1 bp mismatch) the insert is capable of forming stable stem-loop structures as a consequence of the imperfect 15-bp inverted terminal repeat in the *Hinf* element (Fig. 6). This feature is reminiscent of most bacterial transposons and *Drosophila* element FB³⁸ which have longer inverted repeats.

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Specific transcription and RNA splicing defects in five cloned β -thalassaemia genes

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Transcriptional analysis of five different cloned β -thalassaemia genes introduced into cultured mammalian cells revealed specific defects in transcription and RNA splicing. A single base change 87 base pairs to the 5' side of the mRNA cap site significantly lowers the level of transcription and therefore appears to represent a promoter mutation. Three genes contain different single base changes in the first intervening sequence (IVS) 5' splice site. One mutation, at IVS1 position 1, inactivates the splice site completely; the other two, at IVS1 positions 5 and 6, reduce its activity. Each mutation activates the same three cryptic splice sites. The fifth gene contains a single base change within IVS2 at position 745, which results in the formation of abnormal β -globin RNA that contains an extra exon.

THE human globin genes provide a good model for the investigation of genetic disease at the molecular level. Much is known about the structure and function of the globin proteins and the temporal regulation of their synthesis during development (reviewed in refs 1, 2). Both the human α - and β -globin gene clusters have been isolated and the DNA sequences of each gene determined. In addition, the structures of the major RNA transcripts of each gene are known³⁻⁶. This structural information is complemented by a wealth of clinical, genetic and molecular data concerning hereditary abnormalities of globin gene structure and expression^{2,3,5,7}. Such abnormalities may alter the regulation of globin gene expression during development, leading to conditions such as the hereditary persistence of fetal haemoglobin (HPFH). Alternatively, the aberrant expression of individual α - or β -globin genes causes thalassaemias, severe anaemias characterized by imbalanced synthesis of α - and β -globin polypeptide chains.

The β -thalassaemias comprise a group of diseases in which the synthesis of normal β -globin polypeptide is either absent or reduced, designated β^0 and β^+ thalassaemias, respectively. The heterogeneity of these diseases at the clinical level suggested the existence of many different β -thalassaemia alleles, and this prediction has been confirmed by subsequent structural studies. Sequence analysis of β -globin genes from normal and thalassaemic individuals revealed that the β -thalassaemia phenotype is usually associated with point mutations or deletions of a few nucleotides; in rare cases, the third exon of the gene is deleted (reviewed in ref. 7). These mutations are superimposed on a complex pattern of sequence polymorphisms within and surrounding the β -globin gene⁸; in some cases, they may be detected directly in genomic DNA as a result of the alteration of restriction endonuclease recognition sites^{8,9}.

Two different approaches have been used to investigate the effect of thalassaemia mutations on globin gene expression: analysis of RNA synthesis in the erythroid cells of thalassaemia patients, or analysis of the expression of the cloned mutant gene after its introduction into cultured cells on a suitable vector. Both approaches have demonstrated abnormal globin RNA processing in cases of β^{10-17} and α^{18} thalassaemia. Here we analyse the expression of five different β -thalassaemia alleles⁸ after their introduction into cultured cells on vectors derived from SV40¹⁹ and bovine papillomavirus (BPV)²⁰.

β -Thalassaemia alleles analysed

We analysed the expression of five β -thalassaemia (β^{thal}) alleles of known DNA sequence after their introduction into cultured cells. In addition to various common sequence polymorphisms⁸, these genes contain single base changes which may be responsible for the β -thalassaemia phenotype (Fig. 1A): (1) a C $\rightarrow$ G

transversion 87 base pairs (bp) to the 5' side of the mRNA cap site⁸; (2) a G $\rightarrow$ A transition at position 1 of the first intervening sequence (IVS1)⁸; (3) a G $\rightarrow$ C transversion at IVS1 position 5 (S.H.O. and H.H. Kazazian, unpublished results); (4) a T $\rightarrow$ C transition at IVS1 position 6⁸; and (5) a C $\rightarrow$ G transversion at IVS2 position 745⁸.

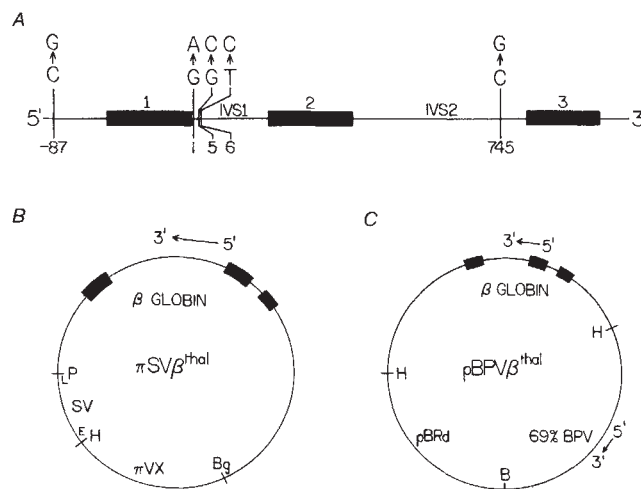


Fig. 1 A, β -thalassaemia genes analysed in this study. The sequences of all genes except the IVS1 position 5 mutant have been described elsewhere⁸. This mutant, which was isolated and sequenced using a previously described strategy⁸, has sequence polymorphisms at exon 1 position 59 C $\rightarrow$ T, and IVS2 positions 16 C $\rightarrow$ G, 74 G $\rightarrow$ T, and 666 T $\rightarrow$ C (S.H.O. and H.H. Kazazian, unpublished data). B, π SV β^{thal} , the SV40-derived plasmids used for expression studies in HeLa cells. These plasmids carry a 3.7-kbp *Bgl*II+*Pst*I fragment containing each of the mutants or a normal β -globin gene inserted between *Bgl*II and *Pst*I sites in the miniplasmid (B. Seed, manuscript in preparation) vector π SVHPplac (P. Little, unpublished). SV, the SV40 *Pvu*II-*Hind*III fragment containing sequences necessary for efficient β -globin gene expression in HeLa cells²¹; π VX, the small tRNA *supF*-containing plasmid constructed by Seed (manuscript in preparation); P, *Pst*I; Bg, *Bgl*II; H, *Hind*III (destroyed in construction of π SVHPplac). The reference plasmid π SVHP α 2 was constructed by subcloning a 1.5-kbp *Pst*I fragment of human DNA containing the human α_1 globin gene into the *Pst*I site of π SVHPplac. C, pBPV β^{thal} , bovine papillomavirus-derived expression plasmids. These plasmids comprise a 7.6-kbp *Hind*III fragment containing the β -globin gene inserted at the *Hind*III site of the vector pBPVH11, which carries the 69% subgenomic transforming *Bam*HI+*Hind*III fragment of bovine papillomavirus²⁰. Polarity of the BPV and globin transcription units is indicated by arrows. H, *Hind*III; B, *Bam*HI.

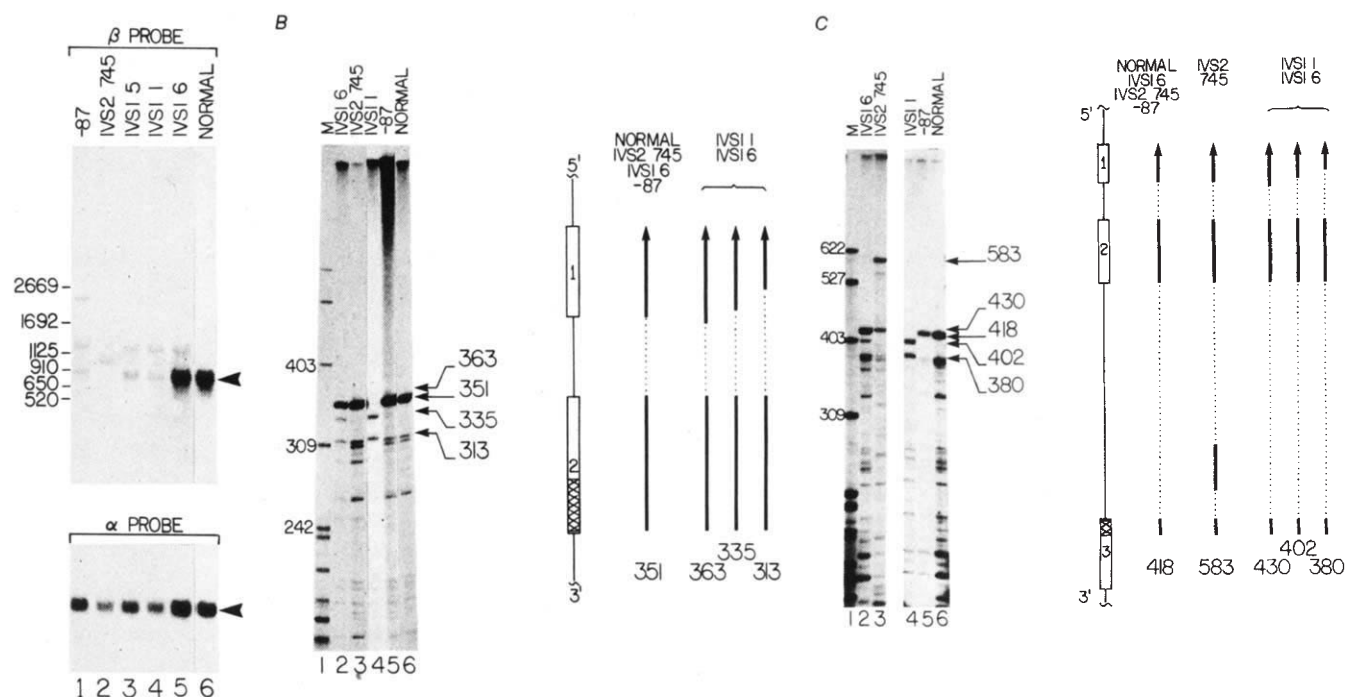


Fig. 2 **A**, β -globin specific RNAs produced by the different π SV β^{thal} plasmids in HeLa cells. 20 μ g of total cellular RNA was fractionated on a 1.4% agarose gel containing formaldehyde, transferred to nitrocellulose and hybridized as described previously⁴⁷ with 32 P-labelled probes specific for either β (upper panel) or α (lower panel) globin transcripts produced by the co-transfected reference plasmid α SVHP α 2. Lanes 1–6, RNA produced by the –87, IVS2 position 745, IVS1 position 5, IVS1 position 1, IVS1 position 6 and normal β -globin alleles, respectively. On long autoradiographic exposure a small amount of apparently normal RNA produced by the IVS2 position 745 mutant was visible. The lengths of fragments of pBR322 DNA run in parallel as size markers are indicated. **B**, Primer extension analysis of polyadenylated cytoplasmic RNA from BPV-globin transfected cell lines using an exon 2 primer (the antisense strand of the *Hae*III + *Bam*HI fragment, comprising nucleotides 210–287, 5'-end-labelled with 32 P). Experimental protocols were as previously described¹⁹. In each experiment, 3 μ g RNA were used, except in the case of the IVS1 position 1 mutant, where 6 μ g were used. The cDNA products were displayed on a 4% polyacrylamide sequencing gel. The 363-nucleotide product is clearly visible on long autoradiographic exposure of the gel (data not shown). Lane 1, *Msp*I fragments of pBR322 DNA as size markers; lanes 2–5, the IVS1 position 6, IVS2 position 745, IVS1 position 1 and –87 mutants, respectively; lane 6, the BPV-globin cell line β 11C, which carries a normal β -globin gene²⁰. A schematic representation of the cDNA synthesis is shown at the right: exons are shown as open boxes with the primer sequences cross-hatched; cDNA products generated in each experiment are shown as arrows. **C**, Primer extension analysis of polyadenylated cytoplasmic RNA from BPV-globin transfected cell lines using an exon 3 primer (the antisense strand of the *Bst*NI + *Eco*RI fragment, comprising nucleotides 6–53, 5'-end-labelled with 32 P). Analyses were performed as above, and the various extension products shown schematically at the right. The 430-nucleotide product is clearly visible on long autoradiographic exposure of the gel (data not shown).

Vectors used to obtain efficient β -globin gene expression

Initially we used a transient assay to investigate the expression of each of the mutant genes, carried on plasmids of the type shown in Fig. 1B (π SV β^{thal} plasmids). These plasmids carry a small segment of SV40 DNA required in *cis* for the efficient expression of cloned β -globin genes in HeLa cells²¹. HeLa cells were transfected^{22,23} with equimolar amounts of each plasmid and a reference plasmid carrying the human α 1-globin gene (plasmid π SVHP α 2; see legend to Fig. 1B) to provide an internal standard for transfection efficiency and RNA recovery. As a control, we performed parallel transfections with a plasmid carrying a normal β -globin gene^{23,24}. RNA was analysed 36–50 h after transfection.

In an alternative approach to the analysis of β^{thal} gene expression, we generated permanent lines of morphologically transformed mouse cells using β -globin gene carrying derivatives of the BPV-derived vector pBPVH11²⁰. Human β -globin genes carried on this vector are maintained extrachromosomally at about 30 copies per cell²⁰. Plasmids carrying each mutant gene (BPV β^{thal} plasmids; Fig. 1C) were used to transform mouse C127 cells and individual foci were picked and grown in mass culture without recloning. Analysis of the DNA in each cell line showed that the majority of the BPV β^{thal} DNA was extrachromosomal and indistinguishable from the input DNA. A variable proportion (up to 30%) of the plasmids consisted

of deletion variants lacking pBRd²⁰ sequences and sequences located to the 3' side of the β -globin gene; however, the gene itself was unaffected (data not shown). The structure of the β -globin RNA produced in these cells is identical to that of RNA produced by globin genes introduced into HeLa cells on the SV40-derived vectors²⁰; we therefore used BPV β^{thal} cell lines as a convenient source of large amounts of β^{thal} globin RNA.

Transcriptional analysis of the mutant β -globin genes

We used a variety of standard RNA mapping techniques to characterize the RNA produced by the mutant genes. The amounts of β -globin-specific RNA produced in HeLa cells by the various mutant genes present were examined by RNA blotting and nuclease S₁ mapping, using the transcripts of the co-transfected α -globin plasmid as a reference standard. We did not attempt to measure the relative levels of RNA produced by the mutant genes in the BPV β^{thal} cell lines, because these lines were not cloned and contained different proportions of nontransformed cells. The spliced structures of the various RNAs were analysed using the primer extension assays shown in Fig. 2. Short radiolabelled single-stranded DNA primers derived from either exon 2 (Fig. 2B) or exon 3 (Fig. 2C) were hybridized to the RNA and extended with unlabelled deoxynucleoside triphosphates using reverse transcriptase. Correctly

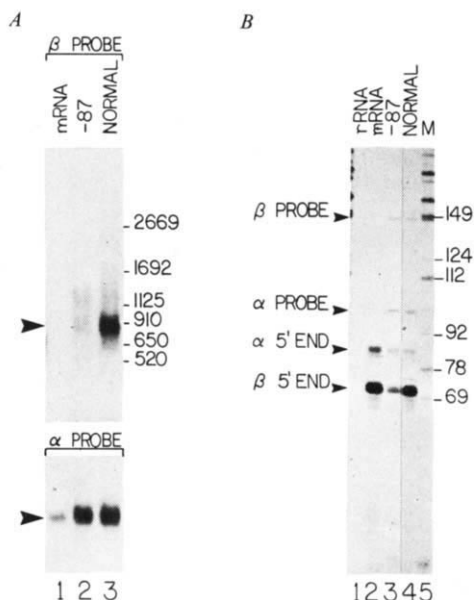


Fig. 3 Transcripts produced in HeLa cells by derivatives of the normal and -87 mutant π SV β^{thal} plasmids containing only 128 nucleotides of β -globin 5'-flanking sequence. These plasmids, constructed by standard procedures, lack all β -globin sequences to the 5' side of an *Rsa*I site at position -128. HeLa cells were transfected with each plasmid plus the reference plasmid π SVHP α 2 which carries the human α_1 globin gene. **A**, Total cellular RNA was analysed by RNA blotting as described in Fig. 2A legend. Hybridization with a β -globin specific probe is shown in the upper panel, and with an α -globin-specific probe in the lower panel. Lane 1, 20 μ g untransfected HeLa cell RNA plus mRNA from human cord blood; lanes 2, 3, 20 μ g of total cell RNA from HeLa cells transfected with the -87 mutant and the normal β -globin genes, respectively. The lengths of pBR322 fragments run in parallel as size markers are indicated. **B**, 5' end analysis of these RNAs by S_1 nuclease mapping⁴⁸. Protocols were as previously described¹⁹, using a mixture of 5' end 32 P-labelled⁴⁸ single-stranded probes specific for β (*Mst*II + *Hae*III, nucleotides -76 to +70) and α (*Bst*NI, nucleotides -19 to +80) globin transcripts. Lane 1, 40 μ g of total RNA from HeLa cells; lane 2, 40 μ g of HeLa cell RNA plus mRNA from human cord blood; lanes 3, 4, 40 μ g of total cell RNA from HeLa cells transfected with the -87 mutant and the normal β -globin gene, respectively; lane 5, marker fragments of pBR322 DNA. Nuclease-resistant fragments mapping the 5' ends of β - and α -globin RNAs (70/71 and ~85 nucleotides respectively), and the intact probes, are indicated.

initiated and spliced β -globin mRNA should yield extension products 351 and 418 nucleotides in length from the exon 2 and exon 3 primers, respectively: these products were indeed generated when polyadenylated cytoplasmic RNA from the BPV-globin cell line β 11C²⁰, which contains a normal human β -globin gene, was used (Fig. 2B, C, lanes 6). Cytoplasmic RNA from HeLa cells transfected with the normal gene also generated these products (data not shown). The predictions of the primer extension assays were confirmed using nuclease S_1 mapping and cDNA sequencing.

The -87 mutant: This β^{thal} gene produces correctly initiated and spliced β -globin RNA, but at a lower level than with the normal gene. Primer extension analysis of polyadenylated cytoplasmic RNA from the BPV β^{thal} cell line V5B1, which carries this gene, is shown in Fig. 2. Primers derived from exons 2 or 3 generated extension products characteristic of correctly initiated and spliced β -globin RNA (351-nucleotide product, Fig. 2B, lane 4; 418-nucleotide product, Fig. 2C, lane 4). Nuclease S_1 mapping confirmed these results, and demonstrated the correct position of the polyadenylated 3' end of these transcripts (data not shown). RNA blotting experiments demonstrated that this gene produces about 10-fold less RNA than the normal

gene in HeLa cells (Fig. 2A, lane 1). The π SV β^{thal} plasmids used in this experiment contained approximately 1.5 kilobase pairs (kbp) of uncharacterized β -globin 5' flanking sequences. To demonstrate that the low level of transcription observed in these sequences, we examined the transcription in HeLa cells of deleted derivatives of the -87 mutant and the normal β -globin gene containing only 128 nucleotides of 5' flanking sequence. Both RNA blotting (Fig. 3A) and nuclease S_1 mapping (Fig. 3B) experiments demonstrated that in this case the -87 mutant again produces about 10-fold less correctly initiated RNA in HeLa cells than the normal β -globin gene. In addition, some larger RNAs were produced at low levels by both genes: we did not characterize these RNAs further. We conclude that the -87 mutation is responsible for the decreased level of correctly initiated RNA.

The IVS1 position 1 mutant: This β^{thal} mutation completely inactivates the IVS1 5' splice site, which leads to the utilization of three nearby cryptic (normally inactive) 5' splice sites. RNA blot analysis showed that in HeLa cells this gene produces some RNA that co-migrates with RNA produced by the normal gene, but at a 10–20-fold lower level (compare Fig. 2A, lanes 4, 6). We analysed the spliced structures of the RNA produced by this gene in the BPV β^{thal} cell line P5C2, using the primer extension assay (Fig. 2B, C). The exon 2 primer generated three abnormal products 313, 335 and 363 nucleotides in length rather than the expected 351-nucleotide product (Fig. 2B, lane 4). Nuclease S_1 mapping showed that the 5' ends of the RNAs were normal, and that the IVS1 3' splice site was active (data not shown); the RNAs that generate these products must therefore be spliced using novel IVS1 5' splice sites. Nuclease S_1 mapping located these splice sites at GT dinucleotides at exon 1 positions 105 and 127 and IVS1 position 13 (Fig. 4A, lane 6; Fig. 4B, lane 8). The exon 3 primer generated products of the lengths expected for RNAs in which IVS2 is excised correctly, while IVS1 is excised using the novel 5' splice sites (380, 402 and 430 nucleotide products, Fig. 2C, lane 4). This conclusion was confirmed by nuclease S_1 mapping experiments (data not shown).

The IVS1 position 5 and 6 mutants: Both of these β^{thal} genes produce normally spliced RNA and three abnormal RNAs identical to those produced by the IVS1 position 1 mutant. An RNA blot analysis of RNA produced by these genes in HeLa cells is shown in Fig. 2A: the position 6 mutant produced almost normal amounts of RNA, and the position 5 mutant about half as much RNA as the normal β -globin gene (compare lanes 3, 5, 6). Primer extension analysis of the structure of the RNAs produced by the IVS1 position 6 mutant in the BPV β^{thal} cell line C3C1, which carries this gene, is shown in Fig. 2. With the exon 2 primer, a prominent 351-nucleotide product characteristic of correctly initiated and spliced RNA was generated; in addition, three new products identical to those generated by the IVS1 position 1 mutant were observed (313, 335 and 363 nucleotide products, Fig. 2B, lane 2). Nuclease S_1 mapping experiments confirmed that these abnormal extension products are specified by RNAs spliced at the same cryptic 5' splice sites as the IVS1 position 1 mutant (compare Fig. 4A, lanes 4, 6; Fig. 4B, lanes 8, 9). Using the exon 3 primer, we demonstrated that IVS2 was correctly excised from all transcripts, generating in addition to the normal 418-nucleotide extension product, products identical in size to those observed in the case of the IVS1 position 1 mutant (compare Fig. 2C, lanes 2, 4). Nuclease S_1 mapping studies of RNA produced in HeLa cells by the IVS1 position 5 mutant revealed that this gene uses the same IVS1 5' splice sites as the IVS1 position 6 mutant; however, in this case a greater proportion of the RNA is abnormally spliced (compare Fig. 4B, lanes 7, 9).

The IVS2 position 745 mutant: This β^{thal} gene produces a major RNA product containing 165 nucleotides of IVS2 inserted between exons 2 and 3 in addition to a small amount of normal β -globin RNA. RNA blotting analysis showed that the gene produces about half as much RNA as the normal β -globin

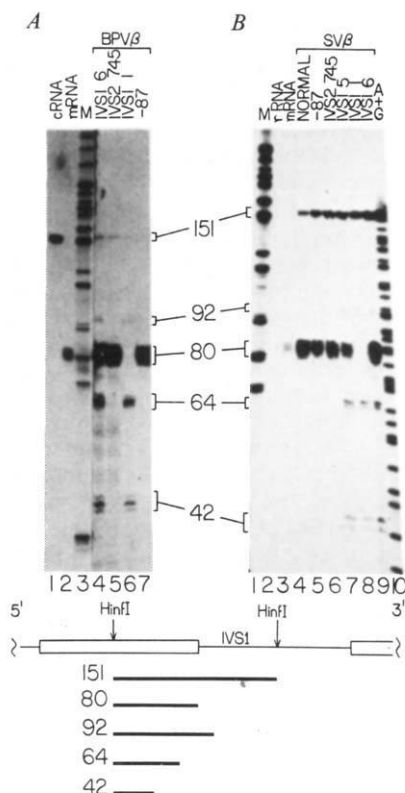


Fig. 4 Nuclease S_1 mapping⁴⁸ of the IVS1 5' splice sites in the normal and mutant β -globin genes, using as probe the 3'-end 32 P-labelled antisense strand of the *Hinf*I fragment spanning nucleotides exon 1 62 to IVS1 68. Experimental protocols were as previously described¹⁹; the results are indicated schematically below the figure. **A**, Analysis of RNA from BPV β^{thal} cell lines. Lane 1, 40 μ g of HeLa cell rRNA plus a continuous transcript of the β -globin gene synthesized *in vitro* (this serves as a control for nuclease sensitivity of continuous hybrids); lane 2, 40 μ g of HeLa cell RNA plus mRNA from human cord blood; lane 3, *Msp*I cut pBR322 size markers; lanes 4–7, 3 μ g of polyadenylated cytoplasmic RNA from BPV β^{thal} cell lines carrying the IVS1 position 6, IVS2 position 745, IVS1 position 1 and $-87 \beta^{\text{thal}}$ alleles, respectively, plus 40 μ g of HeLa cell rRNA. **B**, Analysis of RNA produced by the various π SV β^{thal} plasmids in HeLa cells. Lane 1, *Msp*I cut pBR322 size markers; lane 2, 40 μ g untransfected HeLa cell RNA; lane 3, 40 μ g of HeLa cell RNA plus mRNA from human cord blood; lanes 4–9, 40 μ g total cell RNA from HeLa cells transfected with SV40 plasmids carrying the normal, -87 , IVS2 position 745, IVS1 position 5, IVS1 position 1 and IVS1 position 6 alleles, respectively; lane 10, partial chemical degradation products⁴⁹ of the probe run as homologous size markers, allowing mapping of each splice site to within one nucleotide.

gene in HeLa cells (Fig. 2A, lane 2). Figure 2 shows primer extension analysis of polyadenylated cytoplasmic RNA from the BPV β^{thal} transformed cell line G1C2, which carries this gene. Extension of the exon 2 primer generated a product of 351 nucleotides, indicating correct initiation and IVS1 excision in all transcripts (Fig. 2B, lane 3). These conclusions were confirmed by S_1 nuclease mapping (data not shown). However, extension of the exon 3 primer yielded a predominant product of 583 nucleotides, in addition to a small amount of the 418-nucleotide product characteristic of normal β -globin RNA (Fig. 2C, lane 3). Nucleotide sequence analysis of the 583-nucleotide cDNA product (Fig. 5) showed that it was specified by an RNA containing IVS2 nucleotides 580–744 inserted between exons 2 and 3. Thus, in addition to creating a 5' splice site at IVS2 nucleotide 745, this mutation results in the activation of a cryptic 3' splice site at IVS2 nucleotide 579 (see Fig. 6).

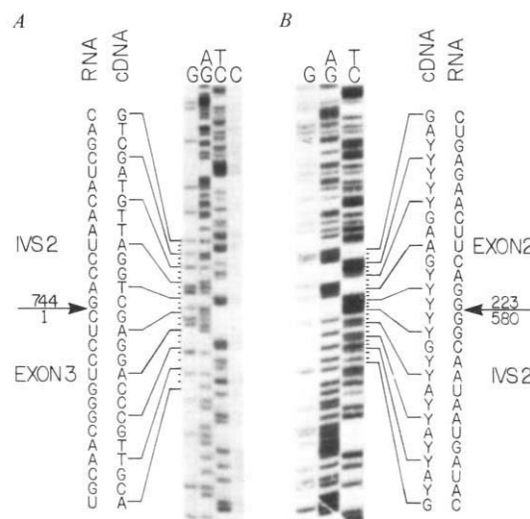


Fig. 5 Sequence analysis of the abnormal transcript produced by the IVS2 position 745 mutant. The primer extension described in Fig. 2C was repeated on a preparative scale using 150 μ g polyadenylated cytoplasmic RNA from the BPV β^{thal} cell line G1C2. The 583-nucleotide cDNA product was subjected to partial chemical degradation⁴⁹ and the products fractionated on 8% (**a**) and 6% (**b**) polyacrylamide sequencing gels. **A**, Sequence across the novel splice joining IVS2 nucleotide 744 to exon 3. **B**, Sequence across the novel splice joining exon 2 to IVS2 nucleotide 580. See Fig. 7 for summary.

The -87 mutation creates a defective promoter

The C $\rightarrow$ G transversion 87 nucleotides 5' to the mRNA cap site seems to be a naturally occurring promoter mutation: in our assay, this gene produces correctly spliced β -globin RNA but at a considerably lower level than the normal gene. Sequences in the -87 to -100 region of several genes are required for efficient RNA synthesis, including those for rabbit β -globin^{25–27}, herpes simplex virus thymidine kinase²⁸ and the SV40 early genes^{29,30}. The -87 mutation lies within the sequence 5' ACACCC 3', which is conserved in both the mouse and rabbit β -globin genes^{4,31} and is necessary for efficient transcription of the rabbit gene when carried on a polyomavirus vector²⁵. Other sequences that have been shown to be required for efficient transcription of the rabbit β -globin gene carried on SV40- and polyomavirus-derived vectors include the conserved 'CCAAT' and 'TATA' elements^{4,32} located 75 and 31 bp to the 5' side of the mRNA cap site, respectively^{25–27}. The recent detection of a β -thalassaemia mutation within the conserved 'TATA' homology³³ suggests that this sequence is also important for RNA synthesis *in vivo*, although the effect of this mutation on transcription has not been studied.

As the gene does produce a small amount of correctly spliced β -globin RNA in our assay, we predict that it represents a β^+ -thalassaemia allele; however, no individual homozygous for the allele has yet been identified.

The IVS1 5' splice site mutations differ in their effects on RNA splicing

The three β -thalassaemia mutations located at the IVS1 5' splice site provide new information regarding the sequence requirements for splicing activity. The requirement for the GT dinucleotide conserved at virtually all 5' splice sites^{34–36} is well established^{18,19,25,36–39}, and the relatively high degree of

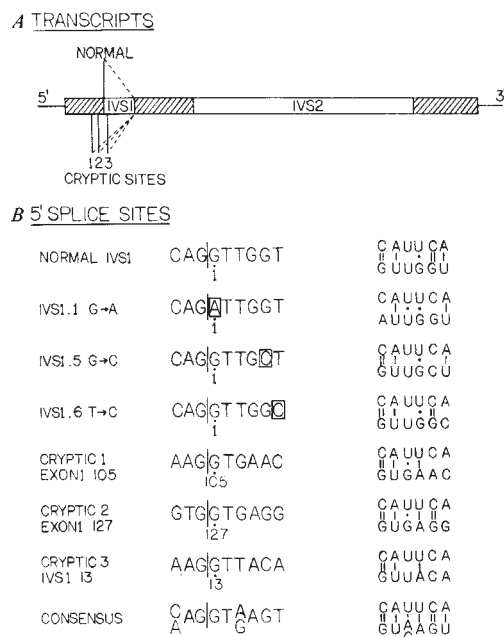


Fig. 6 A, Processing of the IVS1 mutant transcripts. The β -globin gene is shown, with exons represented by hatched boxes and intervening sequences by open boxes; flanking sequences are shown as thin lines. The locations of the normal and cryptic IVS1 5' splice sites are shown by vertical lines above and below the gene. Dashed lines indicate that each 5' splice site is joined to the normal IVS1 3' splice site. **B**, Splice site sequences. In the left column, sequences of the mutant IVS1 and cryptic 5' splice sites are compared with the normal IVS1 5' splice site and the consensus sequence of Mount⁵⁰. Vertical lines indicate IVS 5' boundary according to the GT-AG rule³⁴; the mutations in the IVS1 5' splice site are boxed. In the right column are shown possible base pairing interactions of the first six nucleotides of each intervening sequence with U1 RNA, according to previous models^{43,44}.

sequence conservation at 5' splice sites suggests that the surrounding sequences are important. However, the introduction of purine transitions at positions 3 and 4 of the rabbit β -globin gene IVS2 has no effect on RNA splicing³⁶, while mutation of both nucleotides 5 and 6 of an adenovirus 5 IVS is sufficient to abolish splicing completely⁴⁰. Our results demonstrate that the primary sequence at positions 5 and 6 of IVS1 determines the extent to which the splicing apparatus chooses the correct 5' site for IVS1 splicing from several available potential 5' splice sites.

The IVS1 position 1 mutant does not produce any correctly spliced β -globin RNA in our assays. However, it does synthesize a small amount of aberrantly spliced RNA via the utilization of three cryptic IVS1 5' splice sites, located at exon 1 positions 105 and 127, and at IVS1 position 13 (Fig. 6A). Cryptic splice site activity resulting from the inactivation of a 5' splice site has been observed in several other cases, including a β^0 -thalassaemia allele^{18,19,25,36}. The two IVS1 mutations located at positions 5 (G→C) and 6 (T→C) reduce the efficiency with which the splicing machinery discriminates between the correct IVS1 5' splice site and the three cryptic 5' splice sites nearby that are used by the IVS1 position 1 mutant. This results in the production of the same three abnormally spliced RNAs and in addition, varying amounts of normally spliced β -globin RNA. Interestingly, the cryptic splice site at nucleotide 127 is also activated, independently of the others, by two other mutations: the β^E mutant G→A change at exon 1 position 129 (ref. 41), and a silent T→A change at position 125 (ref. 42) (see Fig. 6B).

Two groups have proposed that the small nuclear RNA U1 acts to align 5' and 3' splice sites by base pairing^{43,44}. The IVS1

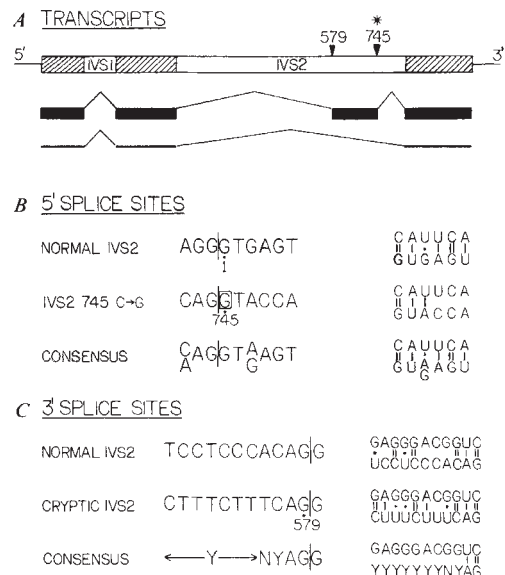


Fig. 7 A, Processing of the IVS2 position 745 mutant transcripts. The β -globin gene is shown, with exons represented by hatched boxes and intervening sequences by open boxes; flanking sequences are shown as thin lines. The locations of the mutation (asterisk) and the cryptic 3' splice site at IVS2 nucleotide 579 are arrowed. Below are shown the two different RNAs produced by the gene in cultured cells: the exons are shown as solid blocks, with splices indicated by carets. **B**, 5' splice site sequences. In the left column, the novel 5' splice site is compared with the normal IVS2 5' splice site and the consensus sequence of Mount⁵⁰. Vertical lines indicate the boundary of the IVS according to the GT-AG rule³⁴. The mutation at IVS2 position 745 is boxed. In the right column possible base pairing between the first few nucleotides of the IVS and U1 RNA is shown, according to previous models^{43,44}. **C**, 3' splice site sequences. In the left column the cryptic 3' splice site at IVS2 position 579 is compared with the sequence of the normal IVS2 3' splice site and the consensus sequence of Mount⁵⁰. Vertical lines indicate the 3' boundary of the IVS according to the GT-AG rule³⁴. In the right column possible base pairing between the last nucleotides of the IVS and U1 RNA is shown, according to previous models^{43,44}.

position 5 and 6 mutations both affect the potential of the IVS1 5' splice site for base pairing with U1 RNA (Fig. 6B), unlike the rabbit β -globin gene IVS2 mutations mentioned above³⁶, thereby providing a rationale for the differing effects of these mutations on splicing. However, although all the cryptic 5' splice sites detected in our experiments can base pair with U1 RNA to some extent, not all sequences that can form base-paired structures of the proposed type with U1 RNA are cryptic 5' splice sites. Similarly, although all the cryptic splice sites we observe are related to the previously defined consensus (Fig. 6B), not all consensus-related sequences form cryptic splice sites. It therefore seems likely that other factors besides primary sequence, such as higher order RNA structure or RNA-protein interactions, must influence splice site activity.

Both the IVS1 position 5 and the IVS1 position 6 mutations are β^+ -thalassaemia alleles, as β^+ -thalassaemia patients homozygous for each allele have been identified (S.H.O. and H.H. Kazazian, unpublished data). This is consistent with our observations that both these genes produce some correctly spliced β -globin RNA in our assays. Furthermore, the severity of the disease observed in these cases correlates with the amount of correctly spliced β -globin RNA produced in our assays: the position 5 mutation causes a more severe anaemia than the position 6 mutation (S.H.O. and H.H. Kazazian, unpublished data). It seems likely that the IVS1 position 1 mutant represents a β^0 allele, as this gene produces no correctly spliced β -globin RNA in our assays.

The IVS2 position 745 mutation creates a new exon

The IVS2 position 745 mutation creates a new GT dinucleotide in a sequence context that produces a new 5' splice site; this in turn leads to the activation of a cryptic 3' splice site at IVS2 position 579. The principal RNA produced by this gene therefore contains an extra exon, comprising IVS2 nucleotides 580–744, inserted between exons 2 and 3; in addition, this gene produces a small amount of normally spliced β -globin RNA (Fig. 7A).

Figure 7B compares the sequence of the new 5' splice site with the 5' splice site consensus and the normal IVS2 5' splice site sequences: it matches the consensus at six out of nine positions, compared with eight out of nine positions for the normal IVS2 5' splice site, and its capacity for base pairing with U1 RNA is less (Fig. 7B). However, it, rather than the normal IVS2 5' splice site, is paired with the IVS2 3' splice site in the majority of the RNA produced by this gene. The cryptic 3' splice site is highly homologous to the 3' splice site consensus (Fig. 7B) and can base pair with U1 RNA (Fig. 7C), and yet is totally inactive in the normal gene. Furthermore, the pairing of splice sites active in this and other IVS2 mutants of the human¹⁹ and rabbit^{25,36} β -globin genes cannot be explained on the basis of a scanning model in which the splicing apparatus binds to one splice site and scans the RNA for a partner⁴⁵. The dramatic effect of this and other single base changes on RNA splicing indicates that such mutations may be instrumental in the evolution of new proteins via the generation of new exons and exon combinations⁴⁶.

Our results predict that the IVS2 position 745 mutant represents a β^+ -thalassaemia allele, because it does produce a small amount of correctly spliced β -globin RNA in our assays; verification of this awaits the identification of suitable β^+ -thalassaemia patients.

Conclusions

The expression of a number of cloned mutant globin genes has previously been examined using cultured cell assays of the type described here^{15,16,18,19,22,25–27,36,41,42}. An important question is whether the behaviour of the genes in these assays is an accurate reflection of their behaviour in the erythroid cell. The cell culture assay does provide a qualitatively accurate picture of

the abnormal splicing events associated with various β -thalassaemia alleles, as several previous studies have established that the abnormal RNA splicing events observed in cultured cells are identical to those observed in erythroid cells from thalassaemia patients (refs 15–19, 22, 40, and R.T. and A. Oppenheim, unpublished results). In addition, we find that a β -thalassaemia allele with a single base change 87 bp 5' to the mRNA cap site exhibits abnormally low transcriptional activity in a cultured cell assay, strongly suggesting that the assay also provides an accurate reflection of promoter activity *in vivo*.

Quantitative comparisons between the amounts of abnormal RNA produced in cultured cells and in the erythroid cells of patients may not be meaningful because it is likely that the rates of synthesis and turnover of these RNAs in the two cell types are quite different. For example, previous studies of the expression of several cloned β -thalassaemia alleles showed that in each case the mutant gene produced the same quantity of RNA as a normal β -globin gene when introduced into cultured cells, while the abnormal RNAs accumulated to only low levels *in vivo*^{15–19,22}. In contrast, all the splicing mutants examined here consistently produced less RNA than the normal gene in our assays: this may be due either to the inefficient processing of these particular mutant precursor RNAs or to the instability of these abnormal RNAs in cultured cells.

In addition to increasing our understanding of the molecular basis of human genetic disease, study of the thalassaemias has led to several conclusions concerning the mechanism of RNA splicing; these can be summarized as follows. The GT and AG dinucleotides at 5' and 3' splice sites are necessary for splicing (IVS1 position 1; refs 18, 19, 25, 36, 37); mutations near GT (or AG?) dinucleotide may result in either a decrease (IVS1 positions 5, 6; ref. 40) or an increase^{41,42} in RNA splicing at these sites. Single base changes that create GT or AG dinucleotides may generate splice sites if in an appropriate sequence context (IVS2 position 745; refs 16, 22). Lastly, mutations which interfere with normal splicing may also lead to the utilization of cryptic splice sites (IVS1 positions 1, 5, 6; IVS2 position 745; refs 18, 19, 25, 36), or generate novel exon combinations¹⁹.

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Runaway instability in accretion disks orbiting black holes

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The runaway instability (very fast, 'catastrophic', mass exchange) operates in close binaries when the more massive star overflows its Roche lobe^{1,2}. The Roche lobe radius shrinks due to the mass exchange more rapidly than the radius of the star. The star keeps overflowing its Roche lobe and continuously loses mass. It has been found³ that a critical equipotential surface similar to the Roche lobe also exists in the black hole accretion disk system. The existence of this lobe is not connected with the gravity of the disk but is due to general relativistic effects in the gravitational field of the black hole alone. We argue here that all the accretion disks which overflow their Roche lobes and have their masses greater than a few per cent of the mass of the central hole are unstable with respect to runaway instability. This may be very important for quasars and other active galactic nuclei.

We shall discuss the runaway instability using a very simple analytical model of the black hole accretion disk system. Our model is very accurate for accretion disks which take the form of slender tori and less accurate for large disks. This slender torus approximation is the only one which limits the generality of the results presented here. We adopt a newtonian model of the black hole⁴ which uses non-relativistic physics and assumes the gravitational potential of the black hole to be of the form

$$\Theta_{\text{BH}} = -\frac{GM}{R - R_G}, \quad (1)$$

$$R_G = \frac{2GM}{c^2} = (\text{gravitational radius})$$

with R being a spherical radius. In this potential the angular momentum of test particles on circular orbits (keplerian motion) is not monotonic, but has a minimum at $R = 3R_G$, exactly as in the case of the Schwarzschild black hole. It has been proved that the pseudo-relativistic potential (1) correctly describes all the relativistic effects which are relevant for the accretion disks around slowly rotating black holes⁴⁻⁶. The very small quantitative differences between the approach based on potential (1) and the correct relativistic one are always less important than the physical uncertainties which are generic to accretion disks (such as the viscosity mechanism).

Let subscripts 'in', 'out' and 'c' refer to the inner and outer edges of the disk and its physical centre (place with maximal pressure) respectively. It is known that for accretion disks which are overflowing their Roche lobe the specific angular momentum l is almost constant close to R_{in} (refs 7, 8). Therefore, if $R_c - R_{\text{in}} \ll R_{\text{out}}$ one can assume that $l = \text{constant}$ between R_{in} and R_c . We shall use here a polytropic relation between the pressure p and density ρ , with γ being the polytropic index: $p = k\rho^\gamma$. The equilibrium formula which describes the balance of the pressure gradient force $\nabla p/\rho$, the gravitational force $\nabla\Theta$, and the centrifugal force, $l^2/R^3 \sin^2\theta$ reads, for the equatorial plane, $\theta = \pi/2$:

$$\frac{(\gamma-1)}{\gamma} \frac{P_c}{\rho_c} = \Theta(R_c) - \Theta(R_{\text{in}}) - \frac{l^2}{2R_{\text{in}}^2} + \frac{l^2}{2R_c^2} \quad (2)$$

So far no approximations, except that $l \approx \text{constant}$ between R_{in} and R_c , have been used.

We use the slender torus approximation only to estimate the total mass of the disk. The mass formula is accurate in the regime $R_c \gg R_c - R_{\text{in}}$. Some support for this approximation follows from detailed numerical models of the radiation pressure supported disks⁹ which show high mass concentration close the circle $R = R_c$ on the equatorial plane. The approximate mass formula reads

$$m = 2\pi^2 \rho R_c (R_c - R_{\text{in}})^2 \quad (3)$$

The location of the physical centre $R = R_c$ is given by the condition that the disk angular momentum must be equal to the keplerian value at the centre

$$l = l_K(R_c) \quad (4)$$

The location of the Roche radius $R = R_0$ is given by a similar condition:

$$l = l_K(R_0), \quad R_0 < R_c \quad (5)$$

The initial configuration fills its Roche lobe that is

$$R_{\text{in}} = R_0 \quad (6)$$

Figure 1 explains the physics of the Roche lobe overflow mechanism and the basic properties of the disk.

We compute the keplerian angular momentum, $l_K = (R^3 d\Theta/dR)^{1/2}$, for two different cases: when the gravitational potential of the disk itself is underestimated

$$l_K = \left[\frac{GMR^3}{(R - R_G)^2} \right]^{1/2} \quad (7)$$

and when it is estimated

$$l_K = \left\{ GMR^3 \left[\frac{1}{(R - R_G)^2} - \frac{m(R_c - R)}{\pi MR_c(R_c - R_{\text{in}})^2} \right] \right\}^{1/2} \quad (8)$$

In the first case $\Theta = \Theta_{\text{BH}}$ that is self-gravity of the disk is neglected. In the second case the internal potential of the slender homogeneous torus is approximated by that of an infinite cylinder with the same density (as in the work of Ostriker¹⁰). The true relation between l_K and R for keplerian distribution lies between those given by equations (7) and (8).

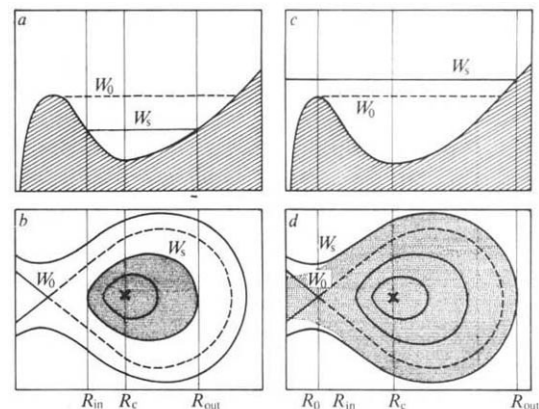


Fig. 1 A typical disk which does not overflow its Roche lobe (a, b) and one which does (c, d). a and c show the potential well; b and d show the meridional section and equipotential structure. Matter distribution is shown by a dark shadow, the Roche lobe by a broken line. On the left, the disk is locked in the potential well: its surface energy, $W = W_0$, is below the top of the potential barrier, $W = W_c$. With the matter supply from the outside, the disk expands and eventually reaches the state in which $W_s > W_0$. Then mass loss through the inner edge $R_{\text{in}} = R_0$ must occur.

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Note that the newtonian approach is sufficiently accurate to compute the gravitational potential of the disk in the case $m \ll M$ as here each dimension of the disk is much greater than its formally defined gravitational radius.

Let us take an infinitesimal mass δM from the disk and put it to the hole. If this is done in an axially symmetric, adiabatic manner then

$$\frac{\delta l}{l} = 0 \quad \frac{\delta p}{p} = (\gamma - 1) \frac{\delta \rho}{\rho} \quad (9)$$

We assume that the disk structure changes homogeneously within the torus which contains most of the mass, that $\delta P_c/P_c = \delta p/p$, $\delta \rho/\rho = \delta \rho_c/\rho_c$. Using these conditions on δl , $\delta \rho$, δp , and perturbed versions of equations (2), (3), (4), one computes the change in the location of the inner radius, R_{in} :

$$\frac{\delta R_{in}}{R_{in}} = A(r_{in}, \mu, \gamma) \frac{\delta M}{M} \quad (10)$$

and using a perturbed version of the equation (5) one gets

$$\frac{\delta R_0}{R_0} = B(r_{in}, \mu, \gamma) \frac{\delta M}{M} \quad (11)$$

Here $r_{in} = R_{in}/R_G$ and $\mu = m/M$.

The explicit formulae for A and B are too long to be presented here. The runaway instability occurs when

$$\frac{\delta R_{in} - \delta R_0}{R_0} = S(r_{in}, \mu, \gamma) = A - B < 0 \quad (12)$$

In the regime $(R_c - R_{in})/R_c = d \ll 1$ (that is, $R_{in} \approx 3R_G$) in which our method is accurate, it follows from the condition (12) that: in the limit $d=0$ all disks are unstable with respect to the runaway instability, independently of the value of μ , and for $d \neq 0$ there are stable disks with $\mu \ll 1$, all other disks being unstable. The astrophysically relevant disk models have R_{in} not too close to R_{MS} . The corresponding d is typically of the order of 0.5. Our method in this region is not very accurate.

The boundary between the stable and unstable regions for $\gamma = 4/3$ in the astrophysically relevant regime is shown in Fig. 2. Note that the curves corresponding to the cases of underestimated and overestimated self-gravity of the disk are very close to each other. This proves that the accuracy in computing the gravitational potential of the disk is not a crucial factor in demonstrating the runaway instability.

The physical nature of the runaway instability in the black hole-accretion disk system is quite different from that in the case of close binaries. The instability occurs in the black hole case because the growing hole changes its gravitational field and therefore the location of the Roche lobe. This is a purely mechanical (dynamical) effect. Therefore the instability acts in the dynamical time scale and the more slow thermal or viscous processes cannot stop it. In the case of close binaries the runaway instability acts in the thermal time scale—it is much slower. There is no dynamical instability connected with the mass exchange in a binary system.

The newtonian model for the black hole does not accurately describe rapidly rotating black holes. If the hole is rotating rapidly and the disk has a strong magnetic field, the hole will lose energy (also mass) and the disk will gain angular momentum¹¹. In this situation the analysis presented here is not adequate and must be substantially changed. One can expect that, instead of runaway, continuous mass loss, something like a sequence of periods with and without mass loss would occur. For the slender self-gravitating rings orbiting slowly rotating black holes there is an analytical, exact, general relativistic solution of the Einstein field equations obtained by Will^{12,13}, which can be used to obtain the same result as given by our model: all the rings, independently of their mass, are unstable

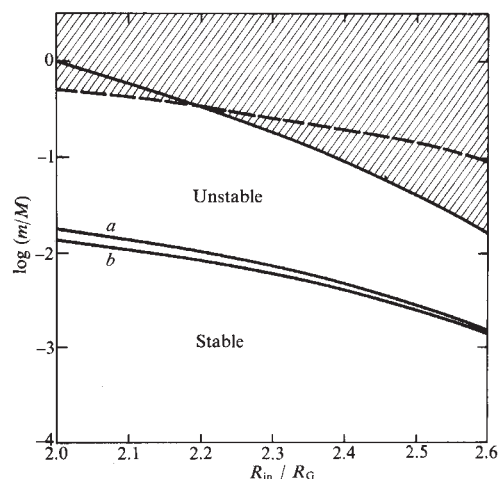


Fig. 2 The runaway instability. Solid curves show the boundary between stable and unstable disk models for $\gamma = 4/3$. *a*, The curve connected with the underestimated potential; *b*, the curve connected with the overestimated potential. Stability with respect to local gravitation fragmentation instabilities¹⁷ changes through the broken line (with stable models being below the line). In the shaded region many of our approximate assumptions are not valid (on the boundary of this region $\delta R_{in}/R_{in}$ is formally infinite for any finite value of $\delta M/M$).

with respect to runaway instability if $R_{in} = R_c = R_{out} = 3R_G$ initially. This supports the use of the pseudo relativistic approach.

We conclude that all accretion disks which overflow their Roche lobes and have shapes which resemble slender tori are unstable with respect to the runaway instability.

Our method is not accurate for thick accretion disks which are much more astrophysically interesting as possible models for quasars and other active galactic nuclei. However, bearing in mind that the more accurate treatment (now in progress) is very difficult and will take a considerable time to complete, let us speculate what our results may suggest for active galactic nuclei assuming that the results shown in Fig. 1 are basically correct.

If this is the case then disks with $m/M > 0.01$ which overflow their Roche lobes are unstable with respect to the runaway instabilities. This instability acts in the dynamical time scale, which can be approximated by the orbital period. The upper limit for it is therefore given by the longest orbital period at the outer edge of the disk; the real time scale may be much shorter:

$$t \ll t_{out} = 3 \times 10^{-4} \left(\frac{M}{10^8 M_\odot} \right) \left(\frac{R_{out}}{R_G} \right)^{3/2} \text{ yr} \quad (13)$$

Even when $R_{out}/R_{in} = 10^6$ this gives only $t_{out} \approx 3 \times 10^5$ yr for the $10^8 M_\odot$ black hole, that is two orders of magnitude less than that which is usually assumed for the active phase of a quasar's life¹⁴.

The luminosity of thick radiation pressure supported accretion disks is implicitly connected with their masses: low mass disks have too small surfaces to radiate at sufficient power. Numerical studies^{9,15,16} show that disks around $10^8 M_\odot$ black hole must have masses of the order $10^7 M_\odot$ to match the energetical demands of quasars. This means that in the active phase of life quasars have $m/M \geq 0.1$. Thus, they must be connected with massive accretion disks which do not overflow their Roche lobes (Fig. 1). Obviously, such disks cannot be stationary as they gain mass from their host galaxies but do not lose mass.

The typical life story of an active galactic nuclei goes through the following sequence, which may repeat many times in the central part of an active galaxy:

Long build up	Short active	Sudden death
with low	→ phase with high	→ due to runaway
luminosity	luminosity	instability

The short active phase is probably connected with energy generation in a process suggested by Paczyński¹⁷: when the density of the disk exceeds $0.1M/R_c^3$ the self-gravity of the disk becomes important (dashed line in Fig. 2) and local instabilities, similar to the Jeans instability develop. If the optical depth is so large that the cooling scale is longer than orbital period the instabilities increase velocity dispersion in the gas. This produces turbulence, thereby enhancing viscous stresses which produce heat and drive accretion. The possibility that active galactic nuclei switch on and off quasi-periodically seems to be consistent with both observation evidence¹⁸ and theoretical ideas¹⁴.

Note that in the case of some exotic galactic objects (SS433, for example^{19,20}) which are conventionally modelled by accretion disks orbiting black holes, one has $m \ll 0.01M$. For such objects, the runaway instability does not work. In fact, the Roche lobe overflow mechanism has in this case a stabilizing effect²¹. More information on the relativistic theory of thick accretion disks can be found in ref. 22, the theory of accretion disks was reviewed recently in refs 23–25.

Finally, let us make another estimation which supports the previously discussed results. The simplest way to show the importance of runaway instability is to discuss a very thin pressureless, non-self-gravitating accretion disk. Such a disk has its inner edge R_{in} at the circle corresponding to the marginally stable orbit for a free test particle orbiting the black hole. It is located at $R = R_{MS} = 3R_G$. The orbits of free particles with $R \leq R_{MS}$ are unstable. Pressureless matter cannot stay on such orbits and must fall down the hole. Let us denote the surface density of the disk by $\Sigma(R)$. Take a small amount of mass δM from the inner edge of the disk and put it into the hole. The change in the location of the inner edge will be $\delta R_{in} = \delta M / 2\pi \Sigma(R_{MS}) R_{MS}$ while the change in the location of the marginally stable orbit is $\delta R_{MS} = \delta M R_{MS} / M$. Obviously, the runaway instability occurs when $R_{MS} + \delta R_{MS} \geq R_{in} + \delta R_{in}$, as in this case some matter is placed on unstable orbits after the mass transfer. This condition is equivalent to

$$\Sigma(R) \geq \frac{M}{2\pi R^2} \equiv \Sigma_{run} \quad (14)$$

for $R = R_{MS}$. Note, that the surface density which corresponds to the equality sign in equation (1) could be of the same order as the one in a disk which is marginally unstable with respect to the self-gravitational fragmentation perturbations of the Jeans type¹⁷, $\Sigma_{BP} = A_s(H/R)M/2\pi R^2$, where H is the disk thickness and the dimensionless parameter A_s is >10 (but its precise value is not known, Paczyński¹⁷ estimates $A_s \approx 16$). The condition for the runaway instability to be more important than the fragmentation instability is $\Sigma_{run} < \Sigma_{BP}$ or $(H/R) \geq (1/A_s) \approx 0.05$. Non-self-gravitating disks models of Muchotrzeb and Paczyński⁷ have $(H/R) = 0.05$ for the critical accretion rate at R_{MS} and $(H/R) > 0.05$ for higher accretion rates at $R = R_{MS}$.

In this simple example all the previously discussed aspects of the runaway instability are clearly visible: (1) runaway instability is important for at least some realistic accretion disk models of active galactic nuclei; (2) both general relativity and self gravity of the disk are important for discussing this instability; (3) the criterion for occurrence of the runaway instability is $\delta R_0 > \delta R_{in}$. Only in the pressureless case it is $R_0 = R_{MS}$, in general $R_0 < R_{MS}$ and this shows that the pressure effects are also important.

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Equatorial confinement of thermal plasma near the rings of Saturn

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In the unique environment of the rings of Saturn, because of a combination of the magnetic field geometry and the meteoroid impact ionization at the ring plane, a thin plasma disk having a total thickness of no more than a few per cent of the planetary radius might exist in the vicinity of the ring system. The radial transport of this ring plasma to larger radial distances may act as an important source of the thermal plasma in Saturn's magnetosphere. I suggest here that the recent observations by the Voyager 2 Plasma Science team are not inconsistent with this scenario.

Because of the extensive nature of the rings of Saturn, important coupling between the ionosphere and the ring plane is expected. For example, the interception of the photoelectron- H^+ pairs from the top-side ionosphere would act as a sink for the ionospheric plasma as well as a source of the neutral atomic hydrogen cloud in the vicinity of the rings¹. Photosputtering² and meteoroid impact³ on the ring plane could also provide a significant supply of neutral atoms and ions in the ring environment. The survival of the neutral atoms and molecules in keplerian orbits and especially, the ions trapped in bouncing motion along the planetary magnetic field, depends largely on the optical depths of the rings and the geometry of the dipole field.

It is possible to store a population of ions with near-equatorially mirroring pitch angles⁴ just under or above the ring plane—that is, if the dipole axis is slightly tilted and offset from the planetary centre as indicated by the Pioneer 11 observations⁵ and the preliminary report of the Voyager finding⁶. A recent re-evaluation of the magnetic field measurements at Saturn by Connerney *et al.*⁷ has indicated that Saturn's planetary magnetic field can be best described by an axisymmetric octupole model. While a tilt of the pointing axis of the magnetic moment from the planetary rotation axis could be ruled out in this new model, a northern offset of the plane of minimum magnetic field by $\sim 0.04R_s$ is still possible. In this sense, the wedge-shaped 'free zone' proposed by Goertz *et al.*⁴ would have to be modified to be a thin slab no thicker than $0.08R_s$ just above the ring plane. The possible presence of such a thin disk of H^+ and O^+ ions is very interesting because the

occurrence of radial spokes of enhanced concentration of fine dust particles in the B ring^{8,9} and the Saturn electrostatic discharge (SED) in the vicinity of the rings^{10,11} might all be related to electrodynamic processes resulting from dust-plasma interaction with the 'free zone' plasma as the main physical ingredient (see refs 4, 12). The confirmation (or otherwise) of its formation could therefore place an important constraint on the development of theories of the Saturn ring-magnetosphere interaction.

As it is not possible to probe the plasma environment of the rings by direct measurements, remote sensing techniques such as radio occultation measurements would be useful, provided the electron density in the 'free zone' is large enough ($n_e \geq 10^3 \text{ cm}^{-3}$). However, a very special edge-on view of the ring plane by the Earth is perhaps required for such spacecraft observations. Thus, only remaining method seems to be *in situ* measurements of the thermal plasma distribution just outside the ring edge at $2.3R_s$.

With the ring system as a strong plasma source of the inner magnetosphere, it is possible in principle to trace the plasma population fed from the free zone. This is because in a rapidly rotating planet, the scale height of an equatorially confined plasma disk could remain almost constant over a wide range of radial distances¹³⁻¹⁵. More specifically, as shown by Siscoe¹⁵, the mirror point latitude λ_m at radial distance r of an ion created at radial distance r_s and mirror point latitude λ_{ms} can be expressed as:

$$\frac{\lambda_m}{\lambda_{ms}} = \left[\frac{(3\xi_s^2 + 2)x}{3\xi_s^2 + 2x^5} \right]^{1/4} \quad (1)$$

where $x = r/r_s$ and $\xi_s = (V_c - V_k)/V_c$ with V_c and V_k as the co-rotating and keplerian velocities at r_s . At $r_s \approx 2.2R_s$, $\xi_s \sim 0.2$; thus for outward diffusing plasma $x > 1$ and

$$\frac{\lambda_m}{\lambda_{ms}} \sim \frac{1}{x} \quad (2)$$

This means that the scale height ($r\lambda_m$) of the ring plasma disk, if extended to a radial distance of $3R_s$ or more, would remain the same in the absence of other external perturbation effects (for example, pitch-angle scattering and charge-exchange loss). It is therefore interesting to check the Voyager 2 plasma observations during the ring plane crossing at $2.88R_s$ (ref. 16) to determine whether there indeed exists a thermal plasma disk confined to the magnetic equator with a scale height of no more than $0.04R_s$. The preliminary report by the Voyager 2 plasma science team¹⁷ has found the scale height of the O^+ (assumed) ions to be $0.2R_s$ at the interval of the ring plane crossing. Such a small scale height, as noted by the experimenters, is not consistent with the thermal temperature measured as 10 eV. One possible explanation¹⁷ is that the observed equatorial confinement is facilitated by the formation of a pancake-like pitch-angle distribution dominated by equatorially mirroring particles. One possible source of such a thin plasma disk is the free-zone plasma transported outwards as a result of radial diffusion.

Due to the presence of tenuous particulate matter distributed in the narrow G ring and the diffuse E ring, the idea of free zone formation might be generalized to regions outside the A ring. In the optically thick parts of the ring system, the time scale of O^+ ion absorption is of the order of a few bounce periods ($t_B \sim 3 \times 10^4 \text{ s}$) if the ring particles are not charged to surface potential $\geq 10 \text{ eV}$. The corresponding loss time scale in the G ring would be $t_A \sim 10^5 t_B$ if the average optical depth of the G ring is taken to be $\sim 10^{-5}$ (ref. 8). The radial diffusion time scales in different regions of the saturnian magnetosphere are still not very well determined. On the basis of Pioneer 11 observations¹⁸⁻²⁰ of the G ring absorption effect of the energetic charged particles, the radial diffusion time scale across the G ring could be estimated to be $t_D \sim 3 \times 10^6 - 3 \times 10^7$ (ref. 21). As $t_D < t_A$, the G ring, even with its mass mainly distributed in a thin layer of only 100 km^{22} , is insufficient to cause the formation of a thin plasma disk confined to the magnetic equator. In view

of this, the observations by the Voyager 2 plasma instrument suggest the existence of a free zone in the vicinity of the main ring system.

There are processes which might lead to decay of the free zone plasma as it diffuses outwards to larger distances. For example, intermixing with the ionospheric plasma could increase the scale height of the thermal plasma disk; Coulomb collision time scale for 10 eV-oxygen ions having a number density of 100 cm^{-3} is $\sim 10^6 \text{ s}$, which is $\leq t_D$. Isotropization of the ring as it diffuses outwards is thus expected. Charge-exchange recombination also could be efficient in producing pitch-angle diffusion. That might be one reason why the thermal plasma at $r \approx 2.88R_s$ was not restricted to the magnetic equator as might have been expected. In any event, the Voyager 2 plasma data apparently do not contradict the idea of equatorial confinement of the ring plasma as a result of the axisymmetric magnetic field^{4,21}.

Frank *et al.*²³ have estimated the source strength of the oxygen thermal ions detected by the Pioneer 11 plasma instrument to be $10^{23} - 10^{24} \text{ ions s}^{-1}$. In comparison, the meteoroid impact ionization has been estimated as being about $10^{27} \text{ ions s}^{-1}$ using the data available²⁴. In principle, feeding of $\sim 0.1\%$ of the impact plasma into the free zone would be sufficient to maintain the thermal plasma population within $5-6R_s$. The potential importance of the ring source of the heavy ions is that it provides an efficient mechanism of electrodynamic coupling between the rings and the entire magnetosphere. As recently proposed²⁵, a two-cell convection system may be established in the vicinity of the D ring as a result of the diurnal modulation of the photoemission rate of the ring particles. If a similar effect exists throughout the whole ring system, the co-rotation pattern of the magnetospheric plasma in Saturn's inner radiation belt might be modified. This point will be considered elsewhere taking into account the pertinent observational data.

Finally, recent studies^{24,26-28} have indicated that the opacity discontinuity at $1.64R_s$ and the inner edge of the B ring at $1.52R_s$ could be related to the equatorial confinement effect of the ring plasma generated by meteoroid impact and ionization of the neutral gas in the vicinity of the ring system. The thermal temperature of impact ions (assumed to be O^+ ions) is of the order of $\leq 10 \text{ eV}$. (E. Grün, personal communication) and the pick-up ions created in the neutral cloud have initial gyro-energies $\leq 2 \text{ eV}$. Thus, with enough energy resolution, plasma measurements such as those carried out by the Voyager PLS instrument could, in principle, elucidate the sources contributing to the thermal plasma disk, and also place important constraints on the ring electrodynamic models²⁴⁻²⁸.

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Pleistocene phosphorites off the west coast of South Africa

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We report here relatively modern phosphogenesis on the continental margin off South Africa, where palaeontologically old limestone (middle Eocene–middle Miocene) has been phosphatized in late Pleistocene times (62–76 kyr) in an area of only moderate and seasonal present-day upwelling^{1,2}. Previously, modern phosphogenesis had been reported as occurring only off Namibia^{3–15}, Peru/Chile^{16–21} and very recently, east Australia^{22,23}. This report, and those describing the late Quaternary east Australia phosphorite^{22,23}, suggest that, contrary to established opinion, strongly enhanced upwelling may not be necessary for phosphorite formation and that oceanic phosphogenic models need not be based exclusively on localized oceanographic regimes such as exist off Peru and Namibia.

Phosphatic limestone occurs on the continental margin off South Africa and Namibia in a broad (~75 km wide) nearly continuous pavement on the southern and eastern Agulhas Bank (Fig. 1), in a narrow zone (~25 km wide) near the shelf-break on its southwestern margin and in extensive but discontinuous patches northwards to Luderitz^{24–28}. Off the Namibian coast to the north of Luderitz, Quaternary concretionary (authigenic) phosphorite^{3–15} and (?) Miocene pelletal phosphorites are found, the former type in association with siliceous, organic-rich ooze^{4,7,8,13–15}. Uranium-series disequilibrium dating of Namibian concretionary phosphate^{9,11,12}, field relationships^{3,29,30} and the occurrence of phosphatic nodules and laminae in various stages of consolidation^{3,5,6,8,10,14} together show conclusively that parts of the Namibian shelf are sites of modern phosphogenesis.

In contrast many phosphatic limestones from the margin south of Luderitz have been dated as Eocene to Pliocene in age using foraminifera, nannofossils, molluscs and brachiopods^{27,28,31–34}. A uranium-series disequilibrium study by Kolodny and Kaplan³⁵ of samples from the Agulhas Bank, to the south-east of our study area, showed that all of the examples studied from that area were older than the limit of the dating method (>800 kyr). Stratigraphical relationships²⁷ and rock-type associations²⁵ also suggest that most phosphatic limestones found off South Africa were phosphatized in two phases, during the late Eocene and either late Miocene–early Pliocene or late Pliocene times^{25,27,33}. The results presented here, however, show that some carbonate phosphatization has occurred as recently as 62 kyr ago.

The phosphorites reported were recovered from the continental margin of South Africa off Cape Town and Saldanha Bay (Fig. 1) at water depths of 350 (sample GB1), between 515 and 365 (sample 310) and 315 (sample 2246) metres. The shelf width in this area varies between 30 and 100 km with the shelf break occurring at depths of about 450 m.

Samples 2246 and 310 are phosphatic non-conglomeratic microfossiliferous limestones (type NI of Parker²⁴ and of Birch²⁸). Nannofossil evidence indicates an early Oligocene to middle Miocene age for the original limestone of sample 310 (ref. 33); and a middle Eocene to middle Miocene age, by

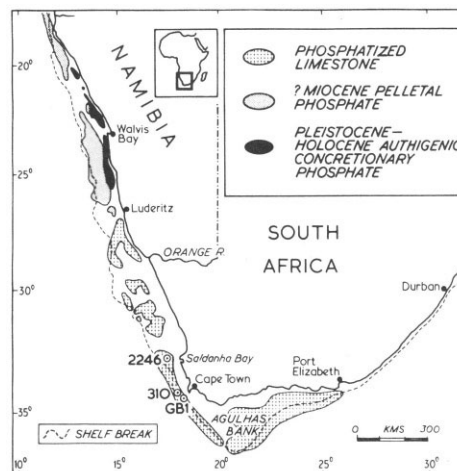


Fig. 1 Distribution of phosphorite on the southern African margin and sample locations.

analogy with nearby dated samples³³, for sample 2246. Sample GB1 is unique among the phosphorites so far recovered from this area. It lacks fossils and comprises 50% well-sorted, very fine sand-size quartz set in a francolite cement or matrix and is described more fully by Birch (ref. 36, Plate IIA p. 96, p. 97). Minerals identifiable by X-ray diffraction analysis are almost exclusively apatite and calcite in sample 2246, apatite and quartz in sample GB1 and apatite, quartz and calcite in sample 310. Tables 1, 2 and 3 present chemical and isotopic data for the three samples together with data for NBS reference material 120b. The chemical data (Table 3) were obtained by plasma emission spectroscopy and atomic absorption analysis; fluorine was analysed by ion electrode and sulphur by reduction to H₂S and iodine titration. The radiometric data (Table 1) were obtained from acid-soluble fractions of the sample powders at Florida State University and from totally dissolved samples at the Institute of Oceanographic Sciences³⁷. The two data sets show good agreement: the small differences in the results obtained are attributable to the different dissolution techniques employed, as the bulk of the thorium (²³²Th) but only a minor amount of the uranium is in the clastic phase. ²³¹Pa/²³⁵U data were obtained by a proxy method in which ²²⁷Th is determined as a measure of ²³¹Pa (refs 37–40).

Table 2 lists the dates estimated from the radiometric data by both the established ²³⁰Th/²³⁴U method¹⁹ and the recently-evaluated ²³¹Pa/²³⁵U method^{40,41}. A correction for ²³⁰Th initially present in the detrital phase has been made in Table 2 based on an initial ²³⁰Th/²³²Th activity ratio of 4.0 (ref. 19). Although a value as high as 4.0 is towards the upper limit suggested by recent work^{40,42}, the resultant corrections to the estimated dates are small. This is a consequence of both the high uranium and low detrital contents of these samples. The correction assumes that ²³⁰Th was introduced into the sample during Pleistocene phosphogenesis. If, instead, all common thorium was introduced during the formation of the precursor carbonate, the contaminant ²³⁰Th would have decayed to insignificant levels since the formation of the carbonate precursor in the late Miocene³³, and the correction would be unnecessary. The ²³¹Pa and ²³⁰Th dates for GB1 would then be substantially more discordant than are the corrected dates.

For samples 2246 and 310 the late Pleistocene ²³¹Pa/²³⁵U and ²³⁰Th/²³⁴U dates agree well. This suggests that a single phase of uranium incorporation has occurred, as two or more phases would be reflected by systematically lower ²³¹Pa/²³⁵U dates relative to the ²³⁰Th/²³⁴U dates because of the different half lives of ²³¹Pa (34.3 kyr) and ²³⁰Th (75.2 kyr). The dates for sample GB1 are not in such good agreement but nevertheless indicate a late Pleistocene period of uranium uptake. It is not clear which, if any, of the dates for sample GB1 is correct although we note that the ²³¹Pa/²³⁵U date agrees well with the dates for the nearby sample 310.

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Table 1 Radiochemical data for South African offshore phosphatic limestones

Sample	Laboratory	U (p.p.m.)	Th (p.p.m.)	$^{230}\text{Th}/^{232}\text{Th}$ activity ratio	$^{234}\text{U}/^{238}\text{U}$ activity ratio	$^{230}\text{Th}/^{234}\text{U}$ activity ratio	$^{231}\text{Pa}/^{235}\text{U}$ activity ratio
NBS-120B	FSU	130±4*	7.9±0.6*	51±2*	1.04±0.01*	0.98±0.03*	0.98±0.04*
NBS-120B	IOS	132±1*	9.1±0.3*	45.0±0.8*	1.05±0.01*	0.99±0.01*	0.97±0.04*
GB-1	FSU	97±3	4.8±0.4	36±2	1.12±0.01	0.53±0.01	—
GB-1	IOS	87.3±0.9*	4.8±0.1*	33±1*	1.10±0.01*	0.53±0.01*	0.72±0.04*
310	FSU	123±4	3.0±0.3	62±13	1.09±0.04	0.45±0.03	—
310	IOS	115±1	4.1±0.2	43.2±0.5	1.10±0.01	0.46±0.01	0.73±0.03*
2246	FSU	269±8	2.4±0.2	180±32	1.10±0.01	0.49±0.01	—
2246	IOS	264±3	1.9±0.1	235±14	1.10±0.01	0.51±0.01	0.78±0.02*

* Weighted mean of more than one analysis. Quoted uncertainties derive from 1 σ counting statistics only.

The CO₂ structurally bound within the apatite mineral in these samples has a $\delta^{13}\text{C}$ value of +0.73‰±0.11‰ (Table 3). This value is squarely within the $\delta^{13}\text{C}$ field of primary marine carbonate (refs 43–45, R. A. Benmore, unpublished results) and confirms the conclusion, based on petrographic study^{25–27}, that the samples are phosphatized carbonates of various types. In addition, the samples are heavier, by about 0.5‰ in ^{13}C and 1‰ in ^{18}O , than other phosphatized carbonates from the margin off South Africa (ref. 45 and R. A. Benmore, unpublished results). This difference is consistent with a supposition that the two sample populations were phosphatized at different times.

These data demonstrate that all three samples are phosphatized carbonates containing recently incorporated uranium. In view of the marked differences in the ages estimated for the original carbonate from fossils and the dates of the phosphatization from uranium-daughter ingrowth, the geochemical validity of the uranium estimates must be examined. The inherent assumptions relevant here¹⁹ are that first, uranium entered the apatite phase at the time of its formation; second, that phosphogenesis was accomplished in a time short in comparison with the estimated age; and third, that apatite has remained a closed system for uranium and its daughter isotopes. That these assumptions are valid for samples 310 and 2246 is shown by several lines of evidence. The similarity in ^{13}C and ^{18}O values between all samples suggests a common origin in time with little subsequent alteration of the apatite. The concordance between the $^{231}\text{Pa}/^{235}\text{U}$ and $^{230}\text{Th}/^{234}\text{U}$ dates would be unlikely if any of the three assumptions were invalid. As the apatite formed by replacement of carbonate it seems reasonable to suppose that the uranium incorporation accompanied the emplacement of P, Sr, Na, F, SO₄ and Mg now present in the apatite phase. Substantial alteration or isotope exchange involving apatite is unlikely to have occurred after its formation not only because of the similarity of the stable isotope results but also because the SO₄/P₂O₅ ratios (59, 64×10⁻³), Na₂O/P₂O₅ ratios (31, 36×10⁻³) and apatite-CO₂ contents (Table 3) are well within the range of other, unweathered, offshore phosphorites from numerous world locations⁴⁶ and substantially above those in weathered samples or in samples that have suffered long term diagenetic alteration^{46,47}. As alteration and

weathering removes these substituents from the apatite structure neither process has affected the samples.

The Sr/P₂O₅ ratios (310 = 93×10⁻⁴; 2246 = 104×10⁻⁴) are above the range for other offshore phosphorites (70–80×10⁻⁴) (refs 46, 48). Sample 2246, with the higher ratio, contains more calcite which suggests that up to 25% of the Sr is present in calcite. Lower than average ratios would be expected if these samples had been substantially altered after formation.

For sample GB1 the above major element ratios (Sr/P₂O₅ 81×10⁻⁴; SO₄/P₂O₅ 61×10⁻³; Na₂O/P₂O₅ 35×10⁻³) and the apatite-CO₂ content (Table 3) are also centrally placed within the range of other offshore, unweathered phosphorites and this suggests that substantial alteration is absent. The discordance between the $^{231}\text{Pa}/^{235}\text{U}$ and $^{230}\text{Th}/^{234}\text{U}$ dates for sample GB1 might, however, be the result of superficial weathering associated with minor secondary enrichment or leaching of uranium or its daughter isotopes. Sample GB1, unlike samples 310 and 2246, has an irregular morphology with burrows or borings which appear to have been enlarged by (?) solution weathering³⁶. If this morphology predates phosphatization, however, no reason for the discordance in dates is readily apparent.

We are satisfied that for samples 310 and 2246, the dates presented here are meaningful geological ages. For sample GB1 the dates are approximate ages. The age range straddles a eustatic transgressive-regressive cycle given by Bloom *et al.*⁴⁹ and suggests that mineralization is not related specifically to high sea-level stands as suggested elsewhere¹⁹. Others have drawn the same conclusion from studies of late Quaternary phosphorite from the eastern continental margin of Australia^{22,23}.

Table 3 Chemical and stable isotopic composition of phosphates

Element (%)	2246	GB1	310
SiO ₂	12.1	45.6	26.7
Al ₂ O ₃	1.95	3.95	3.45
Fe ₂ O ₃ (total)	1.25	2.00	2.30
MgO	0.90	1.00	1.00
CaO	46.0	25.5	37.0
K ₂ O	0.03	0.53	0.64
Na ₂ O*	0.60	0.70	0.72
P ₂ O ₅	11.9	14.6	14.8
CO ₂ †	23.5	5.5	11.2
SO ₄ *	0.90	1.00	1.00
F*	1.66	1.86	1.89
Sr* (p.p.m.)	1,240	1,180	1,380
Total (less O=F and with SO ₄ as SO ₃)	99.8	101.1	99.6
Na ₂ O‡	0.17	0.19	0.26
SO ₄ ‡	0.14	0.11	0.13
CO ₂ §	5.7	4.8	4.9
$\delta^{13}\text{C}\text{‰}$	+0.77	+0.62	+0.80
$\delta^{18}\text{O}\text{‰}$	+3.37	+3.66	+3.78

* Calculated from data on Table 1.

† Corrected ^{230}Th activity used, that is total $^{230}\text{Th} - (4 \times ^{232}\text{Th} \exp(-\lambda_{230}t))$. ^{230}Th and ^{232}Th are activities in d.p.m. g⁻¹, λ_{230} is the decay constant of ^{230}Th , and t is the time elapsed since formation.

* Elements soluble in dilute HCl.

† Total C as CO₂.

‡ Na₂O, SO₄ soluble in distilled water.

§ Structural-CO₂ by Gulbrandsen's method.

Until very recently modern phosphogenesis, and by implication ancient phosphogenesis, was considered by most workers to be a direct result of greatly enhanced coastal upwelling, a restriction which has had considerable influence on the development of phosphogenic models. This report and those describing the late Quaternary Australian phosphorites^{22,23} show that phosphogenesis may occur in areas of only moderate and seasonal upwelling. Bacteria may have a direct role in phosphorite formation²³, in addition to their well-known role in organic degradation which enhances phosphate concentrations in pore-water to very high levels. Contemporary phosphatization may

be possible in areas of moderate upwelling if such non-hydrographic factors have an important role in the process. If this is so, models of phosphorite formation need no longer invoke intense upwelling and necessarily restricted location to explain the formation of ancient phosphorites.

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Uranium disequilibrium dating of phosphate deposits from the Lau Group, Fiji

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Uranium-series methods of absolute age determinations were first applied to fossil coral reefs¹ and more recently to fossil bone², marine apatite^{3–6} and insular apatite^{7,8}. Past sea level stands, climatic conditions, primary productivity, and other factors are thought to be influential in the deposition of avian guano and subsequent insular phosphorite formation⁹. Some deposits appear to have formed during high stands of the sea while others may be related to glacial epochs⁸. We report here an attempt to determine the absolute age of two phosphate deposits in the Lau Group, Fiji by uranium-series disequilibrium techniques, in order to assist in the evaluation of their origin in terms of known palaeoclimatic information.

Phosphatic deposits occur on six islands in the Lau Group—Tuvuca, Vanua Vatu, Ogea Driki, Vatoa, Vatu Vara, and Marabo. The deposits are a combination of phosphatic oolites, pebbles and clay, and lie in the depressions and cracks of the irregular limestone terrain. The phosphates on Vanua Vatu (Fig. 1a) are mainly in the north-west section of the island. The richest concentration of oolites occurs near the school compound where the oolite soil flows like gunshot. Where the oolites are in rock form, they are cemented with a carbonate clay matrix. Some phosphate rock samples were collected from limestone outcrops that stood a meter to several metres above ground. Away from the high concentration areas, the oolites and phosphatic clay decrease in abundance and gradually mix with the red terra rossa soil.

On Tuvuca (Fig. 1b), most of the deposits are clay which fill the solution pits and basins in the interior of the island. Oolitic phosphate occurs in the narrow valleys that rim the island—particularly Nakorovusa and Dranoitiri. Samples used in this study were collected from the limestone walls where the rock phosphate fills seams and solution cavities. The oolites and pebble clasts are cemented with coarsely crystalline calcite. The north end of Nakorovusa slopes down into a small brackish-water pond (Dranolailai) whereas in Dranoitiri, the valley floor is a shallow marsh that dries up during the dry season¹¹.

A maximum age of the Lau Island phosphate occurrences can be determined by the geological age of the underlying Futuna Limestone which occurs throughout the Lau Group. Unfortunately, considerable differences in minimum age estimates have been reported based on the stratigraphical record and faunal assemblages. The range of estimated ages is from lower Miocene¹² to Pliocene (3.9 Myr)¹³ to latest Pliocene. Interruption of reef growth by uplift in the Lau group is estimated to have occurred 3–5 Myr ago, producing the Futuna Limestone (D. W. Woodhall, personal communication). Both phosphate deposits reported on here are within the Futuna Limestone.

Sample mineralogy, P₂O₅ content (in weight per cent) and island locations are listed in Table 1. Mineralogy was determined by X-ray diffraction and phosphate content determined colorimetrically¹⁴. Calcite occurs mainly as a cement and is presumed to be secondary. The uranium content of secondary calcite is usually <1 p.p.m. (ref. 15) and therefore can be considered negligible relative to the uranium content of the phosphatic minerals. Microprobe studies of samples from Vanua Vatu have revealed that the oolites often contain a mineral of apatite composition (high Ca, P, and F) in their centres surrounded by zones which become richer in an aluminium-bearing phosphatic mineral which we assume is crandallite¹¹. Our X-ray results have confirmed its presence in most of our samples. The cement matrix is either a crandallite/geothite mixture or secondary calcite. The pervasive nature of the crandallite and its distribution relative to the apatite indicates that it is probably a replacement of the original apatite. Geothite and small

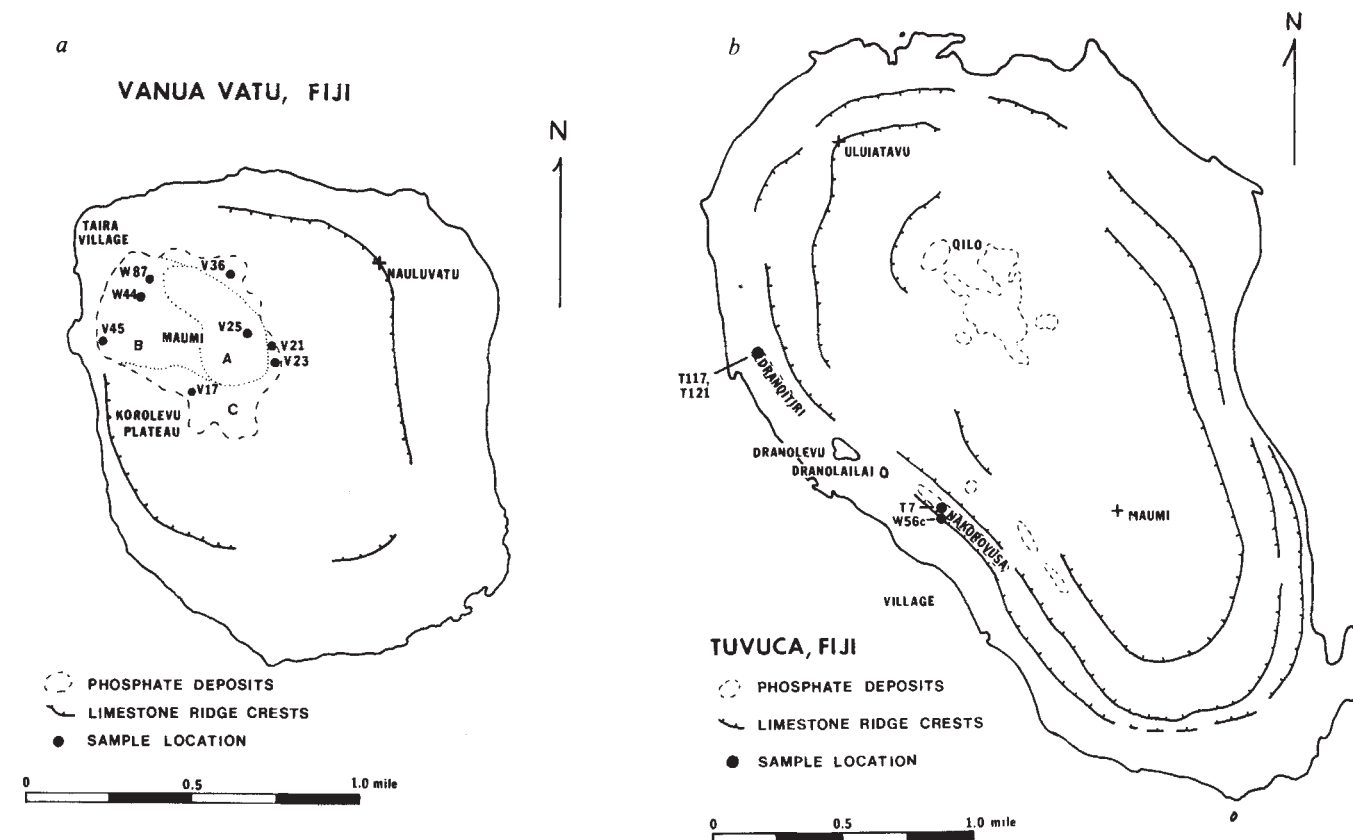


Fig. 1 *a*, Sample location map for Vanua Vatu (after ref. 10). The A, B and C zones were classified¹⁰ as oolitic and pebble phosphate (A), oolitic and pebble phosphate in pockets between limestone outcrops (B), and mostly phosphatic clay (C). *b*, Sample location map for Tuvuca.

amounts of manganese oxides also exist as finely disseminated material within these phosphatic sediments.

Uranium–thorium analyses were performed on the concentrated HNO_3 – HClO_4 soluble phases by isotope dilution α spectrometry following previously described techniques⁶. These

Table 1 Island locations, weight % P_2O_5 , rock type and X-ray mineralogy of Lau Group phosphate samples

Sample	% P_2O_5	Rock type	Mineralogy*
Vanua Vatu (18°22' S, 179°15' W)			
V17	31.66	Phosphatic pisolitic packstone	Ap, Cran
V21	32.88	Phosphatic pisolitic packstone	Ap, Cran
V23	17.50	Phosphatic pisolitic packstone	Cal, Ap, Cran, Whit(?)
V25	28.95	Phosphatic pisolitic packstone	Ap, Cran, Cal
V36	16.73	Phosphatic pisolitic packstone	Ap, Cran
V45	7.10	Phosphatic pisolitic packstone	Cal, Ap, Cran
W44	33.91	Phosphatic pisolitic wackestone	Ap, Cran, Geoth
W87	34.49	Unconsolidated phosphatic pisolites	Ap, Cran, Geoth
Tuvuca (17°40' S, 179°48' W)			
T7	11.56	Phosphatic pisolitic grainstone	Cal, Ap
T117	9.27	Phosphatic wackestone	Cal, Ap
T121	12.18	Phosphatic wackestone	Cal, Ap
W56C	23.44	Unconsolidated phosphatic pisolites	Cal, Ap, Cran, Geoth

Minerals are listed in approximate order of abundance. Those identified by less than three XRD peaks are denoted by question marks.

* Ap, apatite; Cal, calcite; Cran, crandallite; Geoth, geothite; Whit, whitlockite.

samples contained only small quantities of insoluble residues, the range being from 0.4 to 2.4%. An extra 6M NaOH wash after co-precipitation was necessary to remove the high alumina content of these samples. As can be seen in our radiochemical results (Table 2) the usual $^{230}\text{Th}/^{234}\text{U}$ approach to dating does not apply here because of the obvious presence of common thorium ($^{230}\text{Th}/^{234}\text{U}$ activity ratios >1). To determine a valid age based on uranium–thorium disequilibrium the following conditions must hold: (1) the uranium entered the mineral(s) either at the time of formation or shortly thereafter; (2) the deposit was either initially free of ^{230}Th or formed with a constant initial $^{230}\text{Th}/^{232}\text{Th}$ activity ratio in all phases; (3) the deposit has remained a closed system with respect to uranium and thorium isotopes; and (4) the period of deposit formation was short compared with the age to be measured. Since ^{232}Th is present within these samples, it was presumably emplaced with a certain amount of initial ^{230}Th . The age of the host material could be determined by an iterative approach¹⁶ if an estimation can be made of the initial activity ratio $(^{230}\text{Th}/^{232}\text{Th})_0$ of the thorium additions. Otherwise, an isochron method such as applied to igneous rocks^{17–20}, fossil coral terraces²¹, and inorganic carbonates^{22–24} may be applicable if all phases present differ insignificantly in age relative to the half life of ^{230}Th (75,200 yr). In addition, the initial activity ratio $(^{234}\text{U}/^{238}\text{U})_0$ is assumed to be derived from an old limestone source and equal to unity based on the near-equilibrium values measured for all samples. This is unlike sea floor^{4–6} and most insular phosphorites^{7,8}, which form with $(^{234}\text{U}/^{238}\text{U})_0$ similar to the seawater value of 1.14²⁵. If all the above assumptions are met, the age of a deposit, t , can be estimated by using the following relationship:

$$\frac{^{230}\text{Th}}{^{232}\text{Th}} = \frac{^{238}\text{U}}{^{232}\text{Th}} (1 - e^{-\lambda_{230}t}) + \left(\frac{^{230}\text{Th}}{^{232}\text{Th}} \right)_0 e^{-\lambda_{230}t}$$

When $^{230}\text{Th}/^{232}\text{Th}$ is plotted versus $^{238}\text{U}/^{232}\text{Th}$, this equation is in the form $y = mx + b$ with a y -intercept of $(^{230}\text{Th}/^{232}\text{Th})_0 e^{-\lambda_{230}t}$ representing the present-day $^{230}\text{Th}/^{232}\text{Th}$ contamination

Table 2 Uranium and thorium concentrations and activity ratios for Lau Group phosphate samples

Sample	U (p.p.m.)	Th (p.p.m.)	$^{234}\text{U}/^{238}\text{U}$	$^{230}\text{Th}/^{232}\text{Th}$	$^{238}\text{U}/^{232}\text{Th}$	$^{230}\text{Th}/^{234}\text{U}$
<i>Vanua Vatu</i>						
V17	27.7 ± 0.6	1.75 ± 0.11	0.96 ± 0.01	36.5 ± 2.2	47.4 ± 2.6	0.80 ± 0.02
V21	12.2 ± 0.4	1.81 ± 0.09	1.00 ± 0.02	19.2 ± 0.9	20.3 ± 1.2	0.94 ± 0.03
V23	6.8 ± 0.2	1.03 ± 0.05	1.01 ± 0.02	20.3 ± 0.9	20.0 ± 1.1	1.00 ± 0.02
V25	14.0 ± 0.9	1.25 ± 0.16	1.01 ± 0.05	25.6 ± 3.3	33.9 ± 5.1	0.75 ± 0.04
V36	3.7 ± 0.2	1.45 ± 0.14	1.08 ± 0.05	11.1 ± 1.0	7.7 ± 0.9	1.33 ± 0.06
V45	6.6 ± 0.2	1.85 ± 0.13	0.99 ± 0.02	12.4 ± 0.8	10.8 ± 0.9	1.16 ± 0.04
W44	20.2 ± 0.4	1.73 ± 0.07	0.98 ± 0.01	30.8 ± 1.2	35.1 ± 1.6	0.89 ± 0.02
W87	13.7 ± 0.3	1.87 ± 0.09	1.02 ± 0.01	22.3 ± 1.0	22.1 ± 1.1	0.99 ± 0.03
<i>Tuvuca</i>						
T7	2.0 ± 0.1	0.35 ± 0.04	0.98 ± 0.04	16.1 ± 1.6	17.3 ± 2.0	0.95 ± 0.05
T117	4.2 ± 0.1	0.51 ± 0.03	0.97 ± 0.01	24.6 ± 1.4	24.6 ± 1.2	1.03 ± 0.02
T121	4.3 ± 0.1	0.61 ± 0.03	1.02 ± 0.01	21.8 ± 0.9	21.1 ± 1.0	1.01 ± 0.02
W56C	5.3 ± 0.1	1.13 ± 0.04	1.00 ± 0.02	13.0 ± 0.4	14.1 ± 0.7	0.93 ± 0.02

Errors quoted are $\pm 1\sigma$ based on counting statistics.

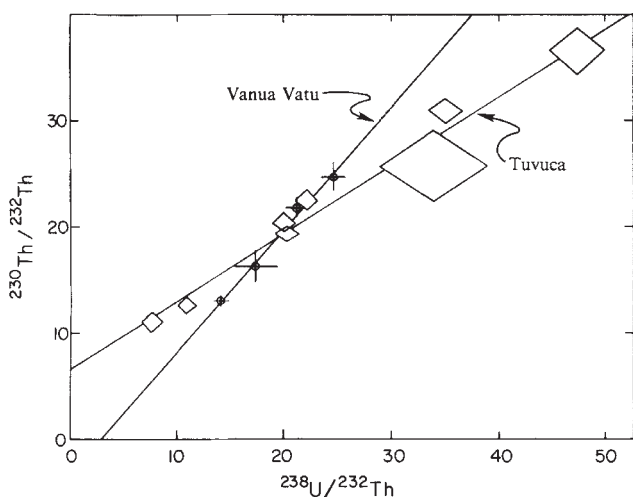


Fig. 2 $^{230}\text{Th}/^{232}\text{Th}$ – $^{238}\text{U}/^{232}\text{Th}$ isochrons for Vanua Vatu (diamonds), and Tuvuca (open circles). Error bars are 1σ counting errors and are not taken into account in the regression analysis. The respective equations and correlation coefficients for the Vanua Vatu and Tuvuca samples are: $y = (0.64 \pm 0.05)x + (6.52 \pm 1.25)$, $r = 0.985$; and $y = (1.15 \pm 0.09)x + (-3.4 \pm 1.8)$, $r = 0.994$.

ratio. The slope of this equation ($^{230}\text{Th}/^{238}\text{U}$) is equivalent to $(1 - e^{-\lambda^{230}\text{Th}t})$ from which an absolute age may be determined.

$^{230}\text{Th}/^{232}\text{Th}$ – $^{238}\text{U}/^{232}\text{Th}$ isochrons for Vanua Vatu and Tuvuca yield $^{230}\text{Th}/^{238}\text{U}$ values equal to 0.64 ± 0.05 and 1.15 ± 0.10 respectively (Fig. 2). The isochron approach works best when sampling includes widely different $^{238}\text{U}/^{232}\text{Th}$ activity ratios. Usually, this can be accomplished by separating the phases into U-rich and Th-rich phases¹⁹, but sampling and analysis of bulk samples containing varying quantities of the same minerals as done here is equally valid. It is also necessary, of course, to work on samples of equivalent age. The strong correlation shown by both our isochrons supports the closed system and equivalent age assumptions.

The age calculated for the Vanua Vatu deposit from the slope of the isochron is $111,000 \pm 15,000$ yr ($\pm 1\sigma$ based on the regression) given the above assumptions. If the source of uranium to the deposit contained excess ^{234}U , that is $(^{234}\text{U}/^{238}\text{U})_0 > 1$, the ^{230}Th produced from the excess ^{234}U must be accounted for¹⁷ and the calculated $^{230}\text{Th}/^{234}\text{U}$ age would be lower. For example, if $(^{234}\text{U}/^{238}\text{U})_0$ was originally equal to the seawater value (1.14) the apparent age would be $92,000 \pm 12,000$ yr. However, since excess ^{234}U decays with a half life of 2.48×10^5 yr, most of the initial excess would still be present in samples approximately 100-Myr old. This is obviously not the case in our samples (Table 2) and supports an initial isotopic composition of the uranium close to equilibrium. However, preferential leakage of ^{234}U relative to ^{238}U is known to occur in sea floor phosphorites and may occur in insular apatite as

well. The leakage of ^{234}U is thought to be related to α recoil displacement^{3,6}. The measured loss rates, however, are at least on an order of magnitude too slow to produce the measured activity ratios in our samples with an initial activity ratio equal to seawater and an age of about 10^5 yr. Initial incorporation of uranium from a remobilized source with $(^{234}\text{U}/^{238}\text{U})_0 > 1.14$ as can occur in lake marls²² would require an even greater apparent ^{234}U leakage rate and therefore is not considered likely.

The 111,000 yr age calculated here centres on a known low eustatic sea level although high stands occur both before and after this glacial period that are within the precision of our measurements^{26,27}. Unlike the Vanua Vatu trend, the Tuvuca Island plot is not distinguishable within 2σ error of an equiline (slope 1.0, intercept 0). Therefore, regardless of the initial uranium activity ratio, we can only assign an age estimate of $>300,000$ yr for this deposit. The samples from Tuvuca are not from a contiguous deposit such as those from Vanua Vatu. The slope (slightly above unity) and negative intercept indicate possible loss of total uranium in one or more samples. With only four samples, the influence of one or two samples on the isochron is large. More samples from Tuvuca should be analysed, therefore, to confirm the age difference between the two phosphate deposits.

The ages of the Vanua Vatu and Tuvuca Islands phosphate deposits indicate two possible periods of phosphorite formation at $111,000 \pm 15,000$ yr BP and $>300,000$ yr BP respectively. Similar ages were identified in sub-lagoonal strata from Mataiva Atoll (Tuamotu Islands) where the isochron dating technique was not needed⁸. Mataiva and Vanua Vatu are the only islands identified thus far with a 110,000–120,000 yr phosphatic strata. If this period was a time of enhanced phosphate deposition throughout the Pacific, other deposits may be present. Most island strata of this age are submergent, however, and the deposits would most likely be buried under a veneer of younger limestone. Phosphatic strata older than 300,000 yr has been previously identified on Nauru, Makatea and Mataiva^{8,28}. The establishment of the absolute ages of these deposits is critical to deciphering the history of insular phosphate formation in the Pacific.

The isochron approach may be appropriate for the determination of absolute ages of other phosphate deposits of late Pleistocene to younger age if the usual $^{230}\text{Th}/^{234}\text{U}$ growth system fails because of the presence of common thorium. We feel that this isochron technique is more realistic than dating models constrained by an assumed value of an initial $^{230}\text{Th}/^{232}\text{Th}$ activity ratio. The method should be valuable although several samples must be analysed in order to discern a good isochron and all minerals containing U must be of insignificantly different age. Recent work on phosphatic concretions and pellets on the sea floor off Namibia (J. Thomson *et al.*, in preparation) and North Carolina (S. R. Riggs *et al.*, in preparation) have produced very encouraging results

demonstrating the wide applicability of the isochron approach. The technique may be particularly useful in very young samples such as those off Namibia because the presence of common thorium is most significant and the initial isotopic composition of uranium has the least effect on the calculated ages.

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Late Proterozoic Brioverian microfossils from France: taxonomic affinity and implications of plankton productivity

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Fossil microbial communities of Precambrian age are divided into two major categories of benthic and planktonic associations. Although some mixing of both categories occurs in transitional inshore and lagoonal biotas, morphological complexity is the distinctive attribute of planktonic associations. Microfossils from late Proterozoic Brioverian rocks (670-640 Myr) in France are comparable to the late Proterozoic microfossil taxa *Sphaerocongregus* and *Bavlinella* which were reported from localities in North America, Scandinavia, Greenland and elsewhere. The microfossils in question are morphologically comparable to certain extant chroococcalean cyanobacterial taxa (*Gomphosphaeria*, *Coelosphaerium*, *Microcystis*). We contend that the fossil microorganisms parallel their supposed extant counterparts not only in that they display externally undifferentiable morphologies, but also in that massive occurrences can in both cases be attributed to eutrophication of waters.

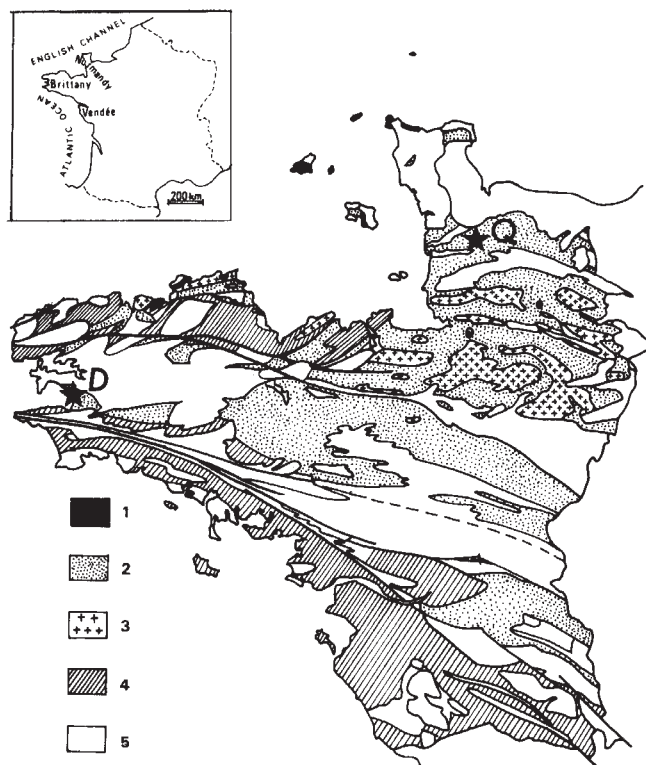


Fig. 1 Cadomian sketch-map of the Armorican Massif. 1, Precadomian basement rocks; 2, Brioverian sedimentary rocks; 3, Cadomian granitoid rocks; 4, Cadomian-Variscan metamorphic rocks; 5, post-Cadomian rock units. D, Douarnenez Bay; Q, quarry at Quibou.

Existing models of Precambrian palaeobiology rely heavily on stromatolitic benthic algal microbiotas and are thus skewed towards a limited sample of pre-Phanerozoic communities^{1,2}. Fossils of Proterozoic planktonic microorganisms, however, occur in sedimentary rocks representing a wide variety of depositional environments^{3,4}, and Proterozoic planktonic biotas are likely to play an increasingly important role in the reconstruction and interpretation of Proterozoic ecosystems^{5,6}.

Upper Proterozoic Brioverian sedimentary rocks in western France (Fig. 1) have yielded rich assemblages of algal microfossils⁷⁻¹¹ which are comparable to microbial microfossils from roughly contemporaneous rocks in North America¹²⁻¹⁴, Scandinavia² and Greenland¹⁵. The present material was recovered from black siliceous rocks (Quibou; Fig. 1) interstratified in a Lower and Middle Brioverian sedimentary sequence. The sequence is intruded by plutonic rocks dated to 670 Myr¹⁶ and is overlain by volcanics dated to 640 Myr¹⁷. Additional material derives from detrital rocks (sub-feldspathic wackes at Douarnenez Bay; Fig. 1) associated with the above mentioned volcanics¹⁸.

The Brioverian fossil microorganism include spherical cell-aggregates consisting of tightly packed spherical cells (Fig. 2). Individual cells are usually less than 1 μm across and the clusters never exceed 8 μm in diameter. The microorganisms are best compared to *Sphaerocongregus variabilis* Moorman 1974 and *Bavlinella faveolata* (Shepeleva) Vidal 1976^{8,9}; possible pleurocapsacean cyanobacterial microfossils reported from North America¹²⁻¹⁴, Scandinavia², Greenland¹⁵ and elsewhere^{2,14}.

Microfossils recovered from rocks at Douarnenez (Fig. 1) consist of clusters of tightly packed cells (Fig. 2A, B). Both clusters and individual cells are hollow and the latter are bound to each other by a membrane (Fig. 2F). Ultrathin sections of specimens recovered from acid-resistant residues reveal under the transmission electron microscope that the cell-clusters consist of compactly arranged individual cells (Fig. 2F). The multi-lamellar structure of the cell wall of individual cells evidently

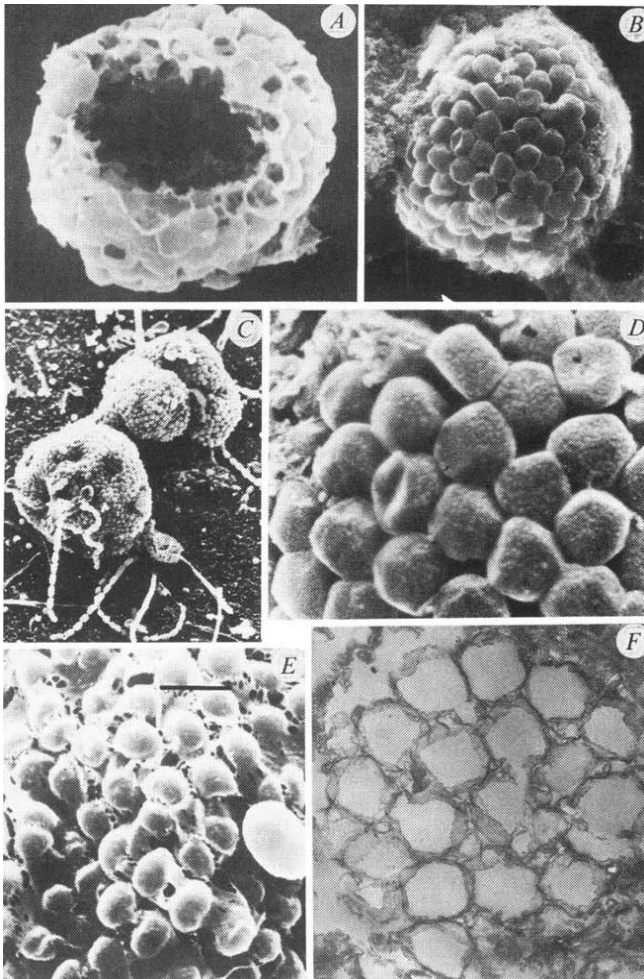


Fig. 2 A, B, Scanning electron micrographs of late Proterozoic, Brioverian, microfossils from Brittany (Baie de Douarnenez, France). The microfossils are cellular aggregates of supposedly chroococcalean cyanobacteria (D is a detail of the central part of the specimen at B) and F is a transmission electron micrograph of internal ultrathin section of a specimen comparable to the specimen at B. C-E, Extant chroococcalean cyanobacteria, *Gomphosphaeria naegeliana*, from Park Lake, British Columbia, Canada. Scale represents E, 1 μm for A, 1.6 μm for B, 40 μm for C, 0.5 μm for D and F, 4 μm for E.

indicates cyanobacterial affinity. The Brioverian microfossils and their above mentioned counterparts do morphologically resemble extant chroococcalean cyanobacteria. Thus, the peripherally arranged cellular aggregates from Brioverian rocks strongly resemble the similarly arranged cell-colonies of *Gomphosphaeria* and *Coelosphaerium* which are supported by a gelatinous matrix. Resemblance is particularly striking with *Gomphosphaeria naegeliana* (Unger) Lemmermann and synonymous taxa (for example, *C. naegelianum*, *Coelosphaerium wichurae* and *Woronichinia naegeliana*). However, the size of individual cells is better corresponded by *C. minutissimum*. Similarity is also found with *Microcystis*, although its cell-colonies usually display a much looser composition than the compactly packed cellular aggregates in our fossil material. Obviously, the above mentioned similarities should not be taken to indicate taxonomic identity between extant cyanobacterial taxa and the fossil material.

Using morphological similarity alone to demonstrate comparable organization could be circumstantial. However, we contend that the resemblance between Brioverian and related fossil forms on the one hand, and supposedly modern counterparts on the other, can be extended to encompass their palaeoecological 'behaviour'. The Brioverian *Microcystis*-like cell-aggregates

(comparable with forms attributed to *Bavlinella faveolata*) are notably abundant in late Proterozoic Varangerian glacial-marine deposits¹⁴. The reason for this has been attributed to explosive blooms in a stressed habitat¹⁴. The Brioverian microfossils derive from offshore turbiditic deposits. They are allochthonous and intrinsically different from Proterozoic stromatolitic benthic microbiotas (which are essentially autochthonous³). Hence, the depauperate taxonomic composition of Brioverian and other late Proterozoic microfossil offshore associations could perhaps be compared with productivity studies of extant plankton. Thus, low biomass and productivity of phytoplankton has been recorded in eutrophicated modern lakes during winter and early spring¹⁹. The greatest productivity rates are, however, accompanied by high cyanobacterial abundances; particularly of *Microcystis aeruginosa*. High levels of dissolved organic and inorganic nutrients in water favour the development of cyanobacteria and cause plankton diversity fall²⁰. We contend that extremely low diversity levels in Proterozoic planktonic associations from turbiditic and glacial-marine sequences mirror the above depicted modern conditions. Furthermore, we claim that these conditions support the above expressed chroococcalean affinity of late Proterozoic microfossils.

The productivity connection suggested here could be erroneously interpreted as being contrary to previously manifested major plankton extinctions in the late Proterozoic⁴. But the available data demonstrate that microfossil assemblages from late Proterozoic interglacial and postglacial (Valdaian) rock units radically differ from immediately younger and older assemblages. This circumstance can not be explained in the light of palaeoecologically connected plankton productivity rates alone. Thus, changes in taxonomic composition of plankton assemblages are observed in a vertical (stratigraphical) scale. These are real, and the individual diagnostic taxa which became extinct without issue do not reappear at a later stage when propitious environments were restored.

A preservational bias of fossil planktonic assemblages might be considered as a possible mechanism favouring cyanobacterial over-representation. However, many investigated fossil assemblages bear no supporting evidence in this direction. Thus, although other microfossil form-taxa are admittedly numerically under-represented their state of preservation is the same as that of supposedly cyanobacterial components. We find that claims of preservational bias cannot be excluded. However, we consider our morphological and plankton productivity model more attractive in that it satisfies both morphological and basic taxonomic representation similarities to present-day conditions.

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Wild potato repels aphids by release of aphid alarm pheromone

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We report here that the aphid *Myzus persicae* (Sulzer) is repelled, at a distance of 1–3 mm, from leaves of the wild potato *Solanum berthaultii* Hawkes. In addition, air from above the foliage induces rapid dispersal of settled aphid colonies, behaviour similar to that of aphids exposed to the aphid alarm pheromone, (*E*)- β -farnesene^{1–4}. The presence of substantial quantities of (*E*)- β -farnesene in air around *S. berthaultii* foliage and in ethanolic washings from it was demonstrated by gas chromatography (GC) and mass spectrometry (MS); the (*E*)- β -farnesene is probably released from glandular hairs on the foliage. We believe that this represents the first time, and certainly for a species related to a major food crop, that a plant has been shown to use the aphid alarm pheromone as an allomone. If this ability could be introduced into cultivated potatoes, it might provide some protection against aphids and hence aphid-borne viruses.

S. berthaultii is a wild tuber-bearing potato, notable for its resistance to a wide range of pests of cultivated potatoes including aphids⁵, leafhoppers⁶, flea-beetles⁷, Colorado potato beetles⁸, mites⁹ and thrips¹⁰. Resistance to all these pests has been associated with the abundance of glandular hairs covering its leaves and stems. The glandular hairs are of two types⁹: the so-called type A hairs have a short stalk and a four-lobed head which ruptures when touched to release a quick-setting fluid, and the type B hairs which exude a sticky droplet at the tip (see Fig. 1). This sticky droplet is not surrounded by a cell membrane and can be washed off with absolute ethanol¹¹.

Type B hairs have not been reported on the cultivated potato, although rudimentary type A hairs occur rarely. *S. berthaultii* plants having both type A and type B hairs are generally more resistant to aphids than plants having type A alone¹⁰. This has been attributed to the combined effects of the type A hairs which, when ruptured, physically trap aphids with their contents and type B hairs which entangle aphids, making them struggle and so rupture more type A hairs¹². However, plants having similar densities and sizes of the two types of glandular hairs may still differ in degree of aphid 'resistance'¹³. This suggested that there might be toxins in the glandular contents or exudates, such as occur in similar hairs of wild tomato¹⁴ and tobacco¹⁵ species. The composition of *S. berthaultii* leaves was therefore investigated to identify the compound(s) responsible for restricting aphid colonization.

Leaves of *S. berthaultii* PI 265858 (from the Plant Introduction Station, Sturgeon Bay, Wisconsin) were washed with absolute ethanol and the extract analysed by capillary column (Flexsil, OV101) GC-MS. Several peaks were observed which were totally or almost absent from a similarly prepared extract of the cultivated potato *Solanum tuberosum* cv. Majestic. Mass spectra indicated the presence of sesquiterpene hydrocarbons (M^+ 204) in the *S. berthaultii* extract. That the compounds were hydrocarbons was confirmed by their immediate elution from Florisil with hexane. Several were tentatively identified from published mass spectra as α -cedrene, cuparene, humulene, β -selinene, β -gurjunene, γ -cadinene, caryophyllene, thujopsene and (*E*)- β -farnesene¹⁶. Although these compounds are known essential oil components of some other plants¹⁷, the presence of (*E*)- β -farnesene is highly significant because it is the main component of the alarm pheromone for most aphid species^{1–4}. The mass spectrum obtained from an authenticated sample of (*E*)- β -farnesene⁴ was similar to that from the *S. berthaultii* extract and identity was confirmed by enhancement of the peak

by co-injection with the authenticated sample on GC with a flame ionization detector (FID) (OV101 Flexsil capillary column). (*E*)- β -farnesene was present at ~300 ng per g of leaf material.

The sticky exudate of type B hairs is readily washed off with absolute ethanol, suggesting this as a source of the (*E*)- β -farnesene. This was confirmed by collecting exudate from several hundred type B hairs on a glass needle: peak enhancement on GC analysis again demonstrated the presence of (*E*)- β -farnesene. In other plants, essential oils are often contained within a glandular structure, for example, the lupulin glands of hops (*Humulus lupulus* L.)¹⁸, so that only traces of components escape into the air above intact plant material. If this were the case for *S. berthaultii*, the (*E*)- β -farnesene, although present, might be present at a concentration insufficient to influence aphid behaviour. This seems to be true for hops⁴, which are now known to contain (*E*)- β -farnesene (J.A.P., unpublished results). Therefore, to determine whether (*E*)- β -farnesene was being released by *S. berthaultii*, air from above intact leaves was passed through pentane to dissolve any volatile hydrocarbons, and the pentane solution was concentrated by evaporation to give a sample suitable for analysis by capillary column GC-FID. Again, the peak for (*E*)- β -farnesene was recorded and its identity confirmed by peak enhancement by adding authenticated (*E*)- β -farnesene to the vessel containing the leaves. Approximately 50 ng of (*E*)- β -farnesene was present in 20 ml of air above 1 g of leaves, a concentration adequate to cause an alarm response¹⁹.

We demonstrated the alarm response as follows. Batches of about 20 young adult apterous *M. persicae* were reared in clip cages on leaves of Chinese cabbage (*Brassica pekinensis*). Five leaflets of *S. berthaultii* or *S. tuberosum* cv. Majestic were placed in a 20 ml syringe and, 5 min later, 10 ml of the enclosed air was expelled from a distance of ~1 cm slowly towards a colony of about 15 newly-moulted adults. The experiment was repeated seven times. Only one of 113 aphids was distributed by air from the syringe containing Majestic leaves whereas 54 of 99 were disturbed by air from the syringe containing *S. berthaultii* (χ^2 test; $P < 0.001$). In a comparable series of experiments, none of 106 aphids was disturbed by 20 ml of air from an empty syringe whereas 20 ml of air containing 50 ng of (*E*)- β -farnesene disturbed 111 of 132 aphids. Aphids



Fig. 1 Type A (a) and type B (b) hairs of *S. berthaultii* B. Scale bar, 95 μ m.

disturbed by air taken from above *S. berthaultii* foliage or by authenticated (*E*)- β -farnesene behaved similarly.

In a second series of experiments consisting of four replicates, adult apterae were placed about 1 cm from leaves of *S. berthaultii* or Majestic, and pointing towards the leaf. Thirty-four of 48 such aphids walked onto Majestic. In contrast, only six of 48 ($P < 0.001$) walked onto *S. berthaultii*, the other aphids turning away, generally at a distance of 1 to 3 mm from the leaf and sometimes subsequently walking parallel to, but 1–3 mm away from, the leaf edge. Similar avoidance behaviour is shown by aphids presented with plants experimentally contaminated with (*E*)- β -farnesene²⁰.

These results demonstrate that *S. berthaultii* foliage, and probably the exudate of type B hairs in particular, releases (*E*)- β -farnesene in amounts sufficient to account for the observed dispersal of settled aphid colonies by air originating around *S. berthaultii* leaves and for the avoidance of leaves by wandering apterae. As well as being a particularly subtle and

fascinating mechanism whereby a plant may be protected against an insect, this phenomenon may have considerable practical significance. *S. berthaultii* is readily hybridized with the cultivated potato, and breeding programmes have already produced hybrids possessing abundant type A and B hairs²¹, making it possible that the ability to release (*E*)- β -farnesene has already been introduced. If so, valuable additional protection against aphids and, more important, against viruses spread by aphids may be obtained. (*E*)- β -farnesene is the alarm pheromone of most pest species of aphids^{1–4}, and alate aphids—probably the morph responsible for most virus spread—are particularly sensitive to (*E*)- β -farnesene²² and avoid treated plants²⁰. In addition, a study of the release system operating in the glandular hairs might suggest approaches to overcome the problem of the instability and high volatility of (*E*)- β -farnesene, which preclude its use for long-term crop protection.

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Denervation increases a neurite-promoting activity in extracts of skeletal muscle

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During early stages of embryonic development, the motoneurons of the spinal cord send out axons that penetrate the differentiating muscle masses and establish connections with individual muscle fibres¹. It has been proposed that during this period the survival, differentiation and axon outgrowth of the motoneurons depend upon retrograde factors produced by the muscles^{2–6}, and in a previous study⁷, we used a quantitative assay for neurite outgrowth from dissociated embryonic spinal neurons *in vitro* to characterize a neurite-promoting activity in media conditioned by embryonic muscle cells. At the adult neuromuscular junction, if some of the axons supplying a muscle are experimentally interrupted, fine nerve processes 'sprout' from the remaining intramuscular nerves and grow to innervate the denervated muscle fibres⁸. In this situation also, it has been postulated that the denervated fibres release a diffusible sprouting stimulus⁹. Using the same *in vitro* assay as before⁷, we now report a striking increase of neurite-promoting activity in extracts of neonatal chick leg muscle after total denervation.

When dissociated spinal neurons from 4½-day chicken embryos were cultured in F12 medium for 20 h, less than 10% of surviving cells developed neurites (Fig. 1A). Parallel cultures performed in the presence of extracts of neonatal chick leg muscle displayed a marked increase in the percentage of cells (up to 40%) bearing one or more neurites after the same period (Fig. 1B). The neurite-promoting activity of a given muscle extract could thus be quantified by constructing a dose-response

curve of the type shown in Fig. 2, the unit of activity being defined as the concentration of muscle extract required to produce 50% of the maximal increase in neurite outgrowth.

The sciatic nerve of 6-day-old chicks (Warren strain) under ether/chloroform anaesthesia was transected at the thigh level and a 3-mm length of the nerve was removed. This operation totally denervated all the muscles of the lower leg and even 3 weeks after operation no functional reinnervation was observed. Other chicks were anaesthetized and sham-operated without transection of the nerve. In all cases, the survival was 100%. Three days later, the chicks were killed and soluble extracts of the muscles of the lower leg were prepared (see Fig. 1) in the following cases: denervated chick (denervated and contralateral legs), sham-operated chick (operated leg) and control untreated chick (one leg). These extracts were assayed for their activity in promoting neurite outgrowth and for their protein concentration (Fig. 2). All the extracts, except that from denervated muscle, had similar specific activities, with a half-maximal effect around 1,500 ng total protein per ml, as reported elsewhere (our manuscript in preparation). In extracts of denervated muscle, however, specific neurite-promoting activity markedly increased, with a half-maximal effect estimated at 100 ng total protein per ml, representing a 15-fold increase in specific activity. These results show that this increase was a specific result of nerve transection, and not a reaction to anaesthesia or muscle wounding. Furthermore, they show that the increase was not systemic but probably localized to the denervated muscle. Such an increase of neurite-promoting activity in the denervated leg with respect to the contralateral leg was reproducibly observed in 12 experiments (Table 1). The mean specific activity ratio of denervated to contralateral muscle extracts was calculated to be 6.0 ± 1.5 (mean $\pm$ s.e.m.; $n = 12$).

The time course of appearance of this activity was next investigated in two strains of chicken, Warren and SV15 Vedette. Starting from a homogeneous batch of 6-day-old chicks in each case, half were denervated by transection of the sciatic nerve and half were maintained as normal controls. Subsequently, two denervated and two normal chicks were killed at different times after operation and the specific neurite-promoting activities (units per mg protein) of their leg muscle

extracts determined independently. The results are shown in Fig. 3. In the case of chicks of the Warren strain (Fig. 3A), there was a steep rise in activity, already detectable 1 day after denervation, with a maximal value at 3 days after denervation. The sudden decrease in activity after this point could be correlated with the visible atrophy (yellowing and stiffening) of the denervated muscles, although no direct relation between the two was established. Using the SV15 Vedette strain (Fig. 3B), the onset of appearance of neurite-promoting activity was delayed, reaching high levels only on the fourth and fifth days after denervation. Little or no atrophy of the denervated muscles of this strain was observed before 10 days after denervation.

A plausible model for the role of a retrograde growth factor in the regulation of synapse formation would be that: (1) non-innervated embryonic target cells synthesize the factor, possibly

in limited amounts, permitting those neurones destined to innervate them to survive and/or differentiate; (2) once the synapses are formed, there is a shut-off of the synthesis of the factor by postsynaptic cells. Accordingly, if these cells are denervated, synthesis of growth-promoting activity will resume until innervation is reestablished. Such indeed would seem to be the case for NGF in the adult rat iris¹¹, where levels of nerve growth factor (assayed by its effect on embryonic ganglia *in vitro*) were increased by at least one order of magnitude 10 days after sensory or sympathetic denervation but fell to undetectable levels concomitant with reinnervation of iris grafts. An effect of denervation on a motor neurotrophic activity in endplate regions of adult mouse soleus muscle has also been reported¹².

In skeletal muscle, observed effects of denervation have been attributed to the action of products of nerve degeneration, to modifications in muscle metabolism brought about by the change in electrical activity¹³, or to both¹⁴. Our results concerning the effect of denervation on neurite-promoting activity do not at present allow us to distinguish between these possibilities. Electrical activity, however, is known to regulate other retrograde phenomena: (1) both naturally-occurring motoneurone

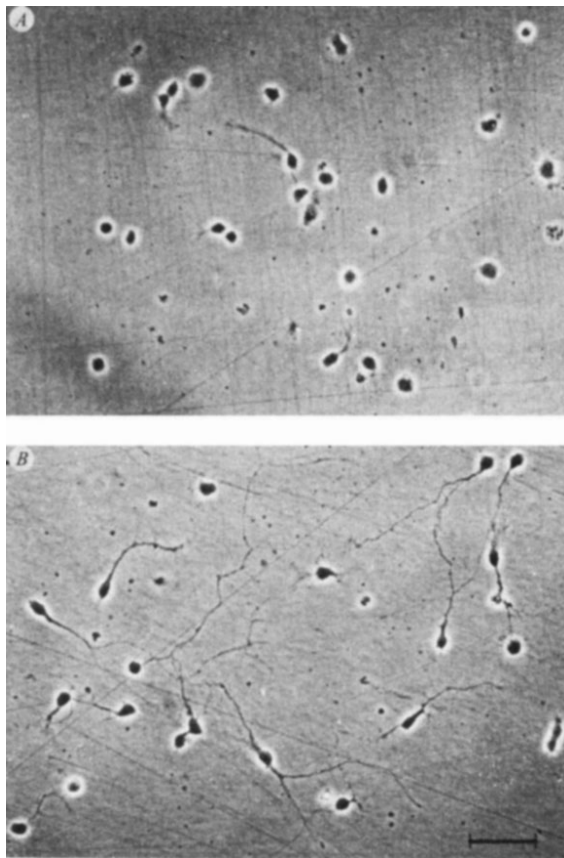


Fig. 1 Effect of muscle extracts on neurite outgrowth in cultures of embryonic chicken spinal neurones. Phase-contrast micrographs are shown of cells cultured for 22 h in: A, F12 medium; B, F12 medium supplemented with an extract of denervated muscle (dilution 1:8,000) at 1 µg total protein per ml. Scale bar, 50 µm.

Methods: Spinal cords were dissected from 4½-day chick embryos (Warren), treated with trypsin and dissociated as previously described⁷. Cells (7×10^4 per well) were plated in 16-mm wells in Costar cluster dishes in 400 µl F12 medium, supplemented with glutamine (2 mM), penicillin (100 U ml⁻¹) and streptomycin (100 µg ml⁻¹). Muscle extracts to be tested were added at the same time. Cells were cultured for 20 h at 37°C in 5% CO₂/95% air and saturating humidity. To prepare soluble extracts of muscle, chicks were killed and the lower leg was freed of skin and bone and finely minced over ice under sterile conditions. To 1 g of muscle was added 4 ml of phosphate buffered saline containing EGTA (3 mM), EDTA (1 mM), aprotinin (5 TIU ml⁻¹), pepstatin (0.5 µg ml⁻¹) and phenylmethylsulphonyl fluoride (0.1 mM) at 0°C, and the tissue was homogenized under sterile conditions using a Polytron homogenizer at half-maximum power for 75 s. Homogenates were centrifuged at 40,000g for 1 h and the clear supernatants collected and tested immediately.

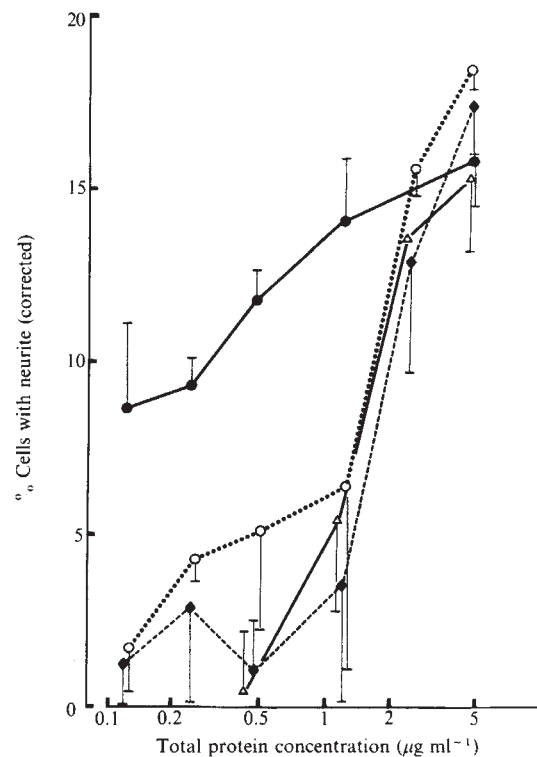


Fig. 2 Dose-response curves using the neurite outgrowth assay for extracts from denervated and control muscles. ●, Denervated chick, denervated leg; ○, denervated chick, contralateral leg; △, sham-operated chick, operated leg; ◆, non-operated chick. Results are expressed as the mean ± range of duplicate assays at each dilution.

Methods: Cells were cultured for 20 h in the presence of a duplicate series of dilutions of the muscle extract. Starting at the perimeter of a well and progressing towards the centre, we counted the total number of cells and the number of these bearing neurites (fine processes > 2 cell diameters long) in 8 adjacent fields. About 250 cells were counted per well; the results are expressed as the percentage of the cells counted that had one or more neurites (% cells with neurite). Values are corrected for the background value of neurite extension in F12 alone, determined for each culture dish (mean value for this set of experiments $7.5 \pm 1.4\%$; s.e.m., $n = 8$). Protein concentrations of muscle extracts were determined by the Folin assay¹⁰ in the linear part of the dilution curve. Denervation (see text) was performed on 6-day old chicks, and extracts were prepared 3 days later.

death¹⁵ and the regression of multiple innervation¹⁶ are retarded when synaptic transmission is blocked; and (2) terminal sprouting in partly denervated muscle is suppressed by direct electrical stimulation⁹.

We consider the neurite-promoting activity described here to be a possible candidate for the role of 'sprouting factor'

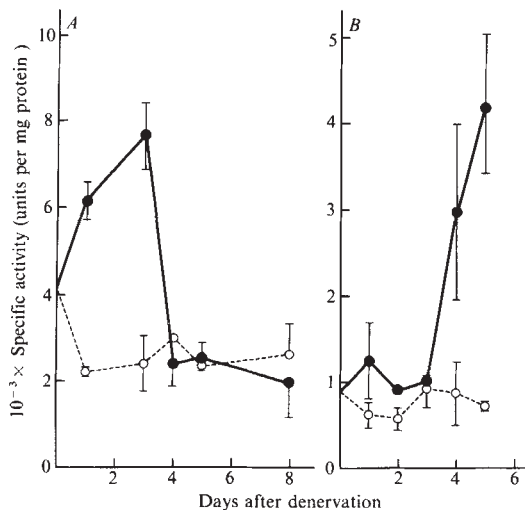


Fig. 3 Evolution with age of the specific neurite-promoting activity in muscle extracts from control and denervated chicks. 6-Day-old chicks from A, Warren or B, SV15, Vedette strains were denervated or maintained as unoperated controls (see text). ●, Chicks denervated at 6 days after hatching; ○, control unoperated chicks. Specific activities (units per mg protein) were determined from dose-response curves of the type shown in Fig. 2. Results are expressed as the mean $\pm$ range of results obtained from two different chicks, but error bars do not take account of the uncertainty in determining the 50% maximal effect from dose-response curves. For practical reasons, specific activity values for 0 days after denervation were supplied from independent experiments on 6-day-old control chicks reared under similar conditions. The total wet weights of muscles in denervated and control chicks of the same age did not in general differ significantly and in no case was a difference observed sufficient to explain the observed effects on specific neurite-promoting activity.

Table 1 Comparison of neurite-promoting activities of muscle extracts from denervated and contralateral legs

Dilution factor	Neurite outgrowth expressed as percentage of value at 2,000-fold dilution ($\pm$ s.e.m.)	
	Denervated	Contralateral
2,000	100	100
4,000	114 $\pm$ 9	71 $\pm$ 5
8,000	91 $\pm$ 6	26 $\pm$ 6
20,000	69 $\pm$ 7	14 $\pm$ 3
40,000	43 $\pm$ 6	6 $\pm$ 3
80,000	36 $\pm$ 9	2 $\pm$ 1

Chicks (Warren) were denervated at 6 days post-hatching by transection of the sciatic nerve. Three days later, muscle extracts were prepared from both denervated and contralateral legs. These were assayed for their activity in the neurite outgrowth assay and dose-response curves of the type shown in Fig. 2 were constructed. Here, results obtained in 12 experiments using extracts from 7 different chicks are combined. Values (% cells with neurite, corrected for F12 background) at each dilution have been normalized with respect to the value in the presence of a dilution of 1:2,000 (v/v) as 100%, and are presented as mean $\pm$ s.e.m. ($n = 6-12$). Absolute mean values (% cells with neurite, corrected) for neurite outgrowth in 2,000-fold dilutions of denervated and contralateral extracts, respectively, were $18.6 \pm 1.1\%$ and $15.9 \pm 1.3\%$. All extracts were prepared in identical conditions at a constant ratio of 0.25 g wet weight of muscle for 1 ml of homogenization medium, and so no allowance was made in the table for the slight variations in total protein concentration of the resulting extracts.

postulated by several authors^{9,17}. Its appearance at an extremely high specific activity (10,000 units per mg protein) is a specific and local response to denervation, with a time course in chick muscles that is comparable to the period (4-6 days^{8,18,19}) that elapses in partially denervated mammalian muscles before the first sprouts are detected.

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Generation of cat retinal ganglion cells in relation to central pathways

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The ganglion cells of the cat retina form classes distinguishable in terms of perikaryal size^{1,2}, dendritic morphology³ and functional properties⁴. Further, the axons differ in their diameters, patterns of chiasmatic crossing and in their central connections⁵⁻⁸. Here we define, by ³H-thymidine autoradiography, the order of production of cells of each class and relate the order of the 'birthdates' to the known axonal pathways. The ganglion cell classes are produced in broad waves, which overlap as cells are produced first for central then for peripheral retina. Medium-sized cells are produced before the largest cells, and small ganglion cells are produced throughout the period of cell generation. This sequence of cell production relates to the orderly arrangement of axons in the optic tract^{9,10}, and can also be related to the rules of chiasmatic crossing observed for each ganglion cell class¹¹⁻¹³.

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Tritiated thymidine (250–500 μCi , specific activity 90–110 Ci mmol^{-1}) was injected into the allantoic sac of 24 fetal cats aged 21–36 days (E21–E36) (see ref. 14 for an evaluation of this procedure). The timed pregnancies were produced by exposing an oestrous female to an experienced male for 12–16 h. The first day of exposure was counted as E0; this differs from the numbering used in earlier summaries^{15–17}, but is the same as that used by others^{18,19}. The animals were killed by perfusion with 10% formol saline after retinal development was essentially complete, 55–260 days after birth. The eyes were removed and the retina and choroid were dissected free, flattened, dehydrated and embedded in glycol methacrylate resin (Sorvall) according to a method described elsewhere (E.H.P. and C.W., in preparation). Sections were cut radially or parallel to the retinal layers at 2–5 μm , then mounted on gelatin-coated slides, processed by standard autoradiographic techniques²⁰ using 14–60-day exposures, and counterstained with cresyl violet. All measurements were taken from sections cut parallel to the retinal layers.

The earliest injections labelled only medium-sized and small ganglion cells in the central retina, and slightly later injections labelled these cells over larger retinal regions. Two experiments with the earliest injections (E21) showed only a few heavily labelled ganglion cells, while many cells were labelled more lightly. The former, which can be interpreted as cells undergoing their final DNA replication at the time of injection²¹, were all small or medium-sized ganglion cells according to the criteria of Stone¹ and Hughes². Scattered in the region of the optic disk and visual streak, they covered a zone having a diameter equal to $\sim 20^\circ$ of visual angle, almost exclusively in the nasal retina. Injections made on days E22–23 produced more heavily labelled medium-sized and small ganglion cells in a larger central region, within $\sim 40^\circ$ of the optic disk (see Fig. 1). Heavy labelling of medium-sized and small cells extended still further towards the periphery when an injection was made on E24.

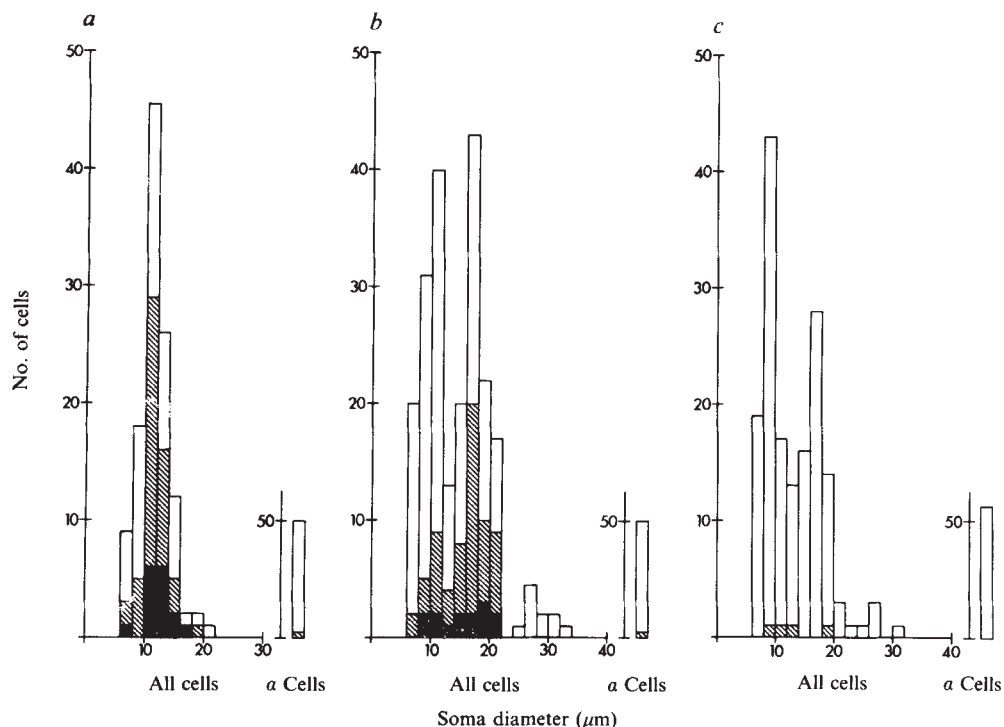
As the region covered by heavily labelled medium-sized and small cells expanded to include the whole retina, heavy labelling among large cells first occurred centrally. Whereas no large cells were heavily labelled in six animals given injections before E25, two ^3H -thymidine injections at E25 heavily labelled a few large cells in the central retina. Two animals given injections on E26 showed heavily labelled small and medium-sized neurones all across the retina, and heavily labelled large neurones in the central retina. These heavily labelled large neurones were widely scattered and located primarily in nasal retina. An injection at E27 labelled still more large neurones in the central retina.

After late injections, labelling among medium-sized cells, and then among large cells, receded from the central retina, became limited to the retinal periphery, and finally disappeared altogether, while labelled small ganglion cells continued to cover the whole retina. Thus, two injections on E28 labelled large and small neurones in the central retina, all cell types at intermediate eccentricities, and medium-sized and small cells in the peripheral retina. Two injections made on E29 produced heavily labelled large and small ganglion cells in all parts of the retina, while labelled medium-sized ganglion cells were restricted to the periphery (see Fig. 2). Two animals given injections on E31 had no labelled large cells $<10^\circ$ temporal to the area centralis, or $<35^\circ$ nasal to the area centralis. Medium-sized cells were labelled only near the ora serrata, while small cells were labelled all across the retina.

Injections made later than E31 (E35, 36) labelled only small cells. Some of these were small ganglion cells according to the criteria of Stone¹ and Hughes², indicating that these cells are still generated after production of medium-sized and large ganglion cells is complete. However, we have not determined the age at which ganglion cell production ceases.

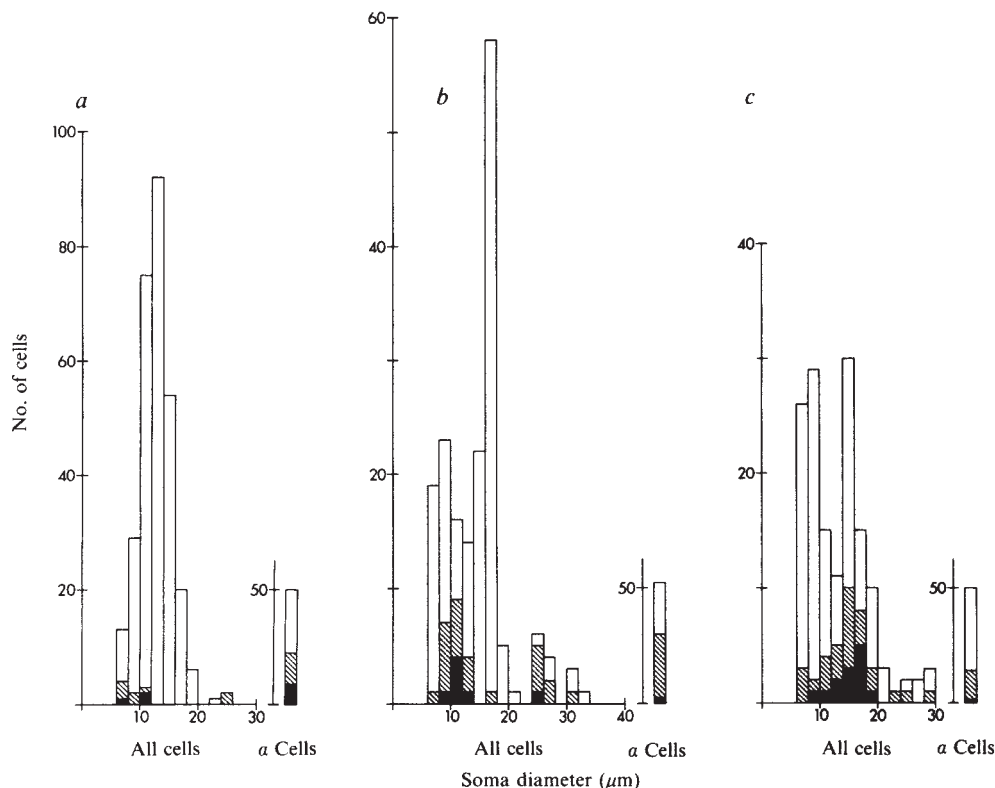
These results show the order in which those ganglion cells that survive in the adult are produced. The results do not suggest

Fig. 1 Size distributions of labelled ganglion cells from an animal given 500 μCi of ^3H -thymidine on embryonic day 23 and killed 182 days after birth. Heavily labelled cells (shown by solid bars) are exclusively small or medium-sized, as are most lightly labelled cells (cross-hatched bars). Open bars represent unlabelled cells. Labelled cells are common in central regions of the retina (*a*, *b*) but rare in the periphery (*c*). Large (α) cells are occasionally lightly labelled, suggesting that they are formed rather later. Each histogram was prepared from three or four serial 2- μm sections from: *a*, within 500 μm of the area centralis; *b*, 1.5 mm temporal to the area centralis; and *c*, 9 mm temporal to the area centralis. Results from nasal retina were similar, although nasal retina developed earlier than temporal retina. All cells with visible Nissl substance and that had a well-defined, pale nucleolus in the plane of the section were included. Soma diameter was defined as the mean of the greatest and least diameters.



Duplicate cell counts were eliminated. Heavily labelled cells contained over half the number of grains seen in the most heavily labelled nucleus in the section, and lightly labelled cells contained one-eighth to one-half the maximum number of grains but significantly above background levels. The method of ^3H -thymidine delivery used here produces a pulse of label with an extended tail, falling to $<50\%$ of maximum after 4 h, and $<10\%$ after 24 h⁹. Because low levels of ^3H -thymidine may be available long after the injection, all cells with less than one-eighth the maximum number of grains were treated as unlabelled. As the largest neurones (α cells) represent $<5\%$ of the total neurone population, separate samples of at least 50 large neurones (no duplicates) were taken from larger regions which included the area in which all neurones were counted. The fraction of the total nuclear volume included in a section, and thus the intensity of label, is inversely proportional to nuclear size⁴². Appropriate corrections have been computed¹⁶, but as they do not significantly affect the cell distributions in these histograms, the uncorrected data are presented.

Fig. 2 Histograms prepared from three or four serial 2- μ m sections: *a*, within 500 μ m of the area centralis; *b*, 3 mm temporal to area centralis; *c*, 9.5 mm temporal to area centralis, from an animal that received ^3H -thymidine on embryonic day 29 and was killed 206 days after birth. All conventions as in Fig. 1. Ganglion cells were labelled across the entire retina. In central regions, the labelled neurones were small or large (*a*, *b*) while in peripheral retina, medium-sized neurones were also labelled (*c*). Note also that while some large ganglion cells were labelled all across the retina, the proportion of large cells that was labelled varied with retinal location.



differential cell death during development^{22,23} nor do they differentiate between displaced amacrine cells and ganglion cells. However, the results do show, as do comparable results summarized by others¹⁹, that in the cat the ganglion cells are not produced in a sequence that is in accord with the retinotopic maps formed centrally by the axon terminals (see also refs 21, 24, 25). Thus, cells whose axons have essentially the same terminal sites can be generated at widely differing times (for example, large and medium-sized cells form in register maps in the same geniculate layers), and cells born at the same time can have widely differing terminal sites. In this respect, the development of the mammalian retina differs from that of amphibians and fish, in which growth occurs mainly by a continuous addition of rings of new cells to the retinal periphery throughout post-embryonic life²⁶⁻²⁸. This pattern of growth allows the animal to maintain a fully functional retina while new cells are being added (S. Easter, personal communication) and also produces an order of addition of ganglion cells that represents a single retinal map. The difference in the pattern of cell addition between non-mammalian and mammalian forms may, therefore, reflect the differing functional demands on the developing retina.

Despite these differences between the cat and non-mammalian forms, fibre order within the optic tract reflects the order of ganglion cell production in all species examined^{16,29-33}. The order of ganglion cell production that we have found in the cat retina is closely related to fibre order in the optic tract if one relates ganglion cell size to axonal diameter in terms established by others⁵⁻⁷. Thus, in the cat optic tract, medium-calibre axons lie deep in the tract, furthest from the pia, and represent the oldest ganglion cells. Large and small axons lie nearer the pia, and a dense group of finest axons is in a sub-pial position, representing the last addition of small ganglion cells^{9,10}. Moreover, just as ganglion cells of each size class are produced in a rough central-peripheral sequence, so fibres of each size class are roughly mapped in the tract, with axons from the central retina lying generally deeper in the tract than axons from peripheral retina. The oldest retinofugal fibres can be labelled preferentially by an intraocular injection of anterograde tracers during the period of ganglion cell production^{34,35}. This experiment has been done in the ferret, also a member of the order Carnivora with a visual pathway similar

to that of the cat and other carnivores^{36,37}, and the results show that the segregation in the tract of older from newer fibres is quite sharp, and is maintained throughout most of the length of the tract³⁸.

The pattern of cell generation defined by our results relates significantly to the pattern of chiasmatic decussation for the axons of each cell type. It has been shown that medium-sized ganglion cells, which are among the earliest to be produced, have axons with the sharpest line of decussation, so that in the retina there is minimal spatial overlap between cells having crossed axons and those having uncrossed axons¹¹⁻¹³. The axons of large ganglion cells, which are produced later, have a line of decussation that is further temporal, and these cells show more 'naso-temporal overlap'¹¹⁻¹³. There is evidence that the small ganglion cells of the temporal retina that project contralaterally are the last ganglion cells to be produced in the temporal retina, as their axons run close to the surface of the tract near the pia^{9,10}. The possibility that there is more than one wave of small cell production, and more than one functional class of small cells³⁹⁻⁴¹, remains to be explored in detail. It seems that, as the retina develops, the factors that produce a sharp naso-temporal segregation become weaker, and their effect moves nearer to the temporal pole, so that progressively more of the axons arising in the temporal retina take a crossed path and the line of decussation becomes less clearly defined.

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Synapse elimination in neonatal rat muscle is sensitive to pattern of muscle use

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The synaptic connections among the cells of the vertebrate nervous system undergo extensive rearrangements early in development^{1,2}. During their initial growth, neurones apparently form synaptic connections with an excessive number of targets, later retracting a portion of these synapses in establishing the adult neural circuits. Because of the profound effects which experience has upon the developing nervous system³, a question of considerable interest has been the role which the functional use of these developing synapses might play in determining the final pattern of connectivity. At the neuromuscular junction the early changes in synaptic connections are well documented, and here questions about the importance of function can be relatively easily addressed. Mammalian skeletal muscle fibres experience a perinatal period of synapse elimination so that all but one of several synapses formed on each muscle fibre are lost⁴⁻⁶. This synapse elimination is sensitive to alterations of neuromuscular use or activity. Reduction of muscle use by tenotomy^{7,8} or by paralysis of the muscle with drugs blocking nerve impulse conduction⁹ or neuromuscular transmission¹⁰ delays or even prevents synapse loss, while increased use produced by stimulation of the muscle nerve¹¹ apparently accelerates the rate at which synapses are lost. I report here a further examination of the role of neuromuscular activity in synapse elimination. I show that chronic neuromuscular stimulation accelerates synapse elimination but that this acceleration is dependent on the temporal pattern in which the stimuli are presented: brief stimulus trains containing 100 Hz bursts of stimuli produce this acceleration whereas the same number of stimuli presented continuously at 1 Hz do not. Furthermore, the 100 Hz activity pattern which is effective in altering synapse elimination also alters two other muscle properties: the sensitivity of the muscle fibres to acetylcholine and the 'speed' of muscle contractions. These findings suggest that the ability of muscle fibres to maintain more than one nerve terminal, like other muscle properties, is sensitive to the pattern of muscle use rather than just the total amount of use.

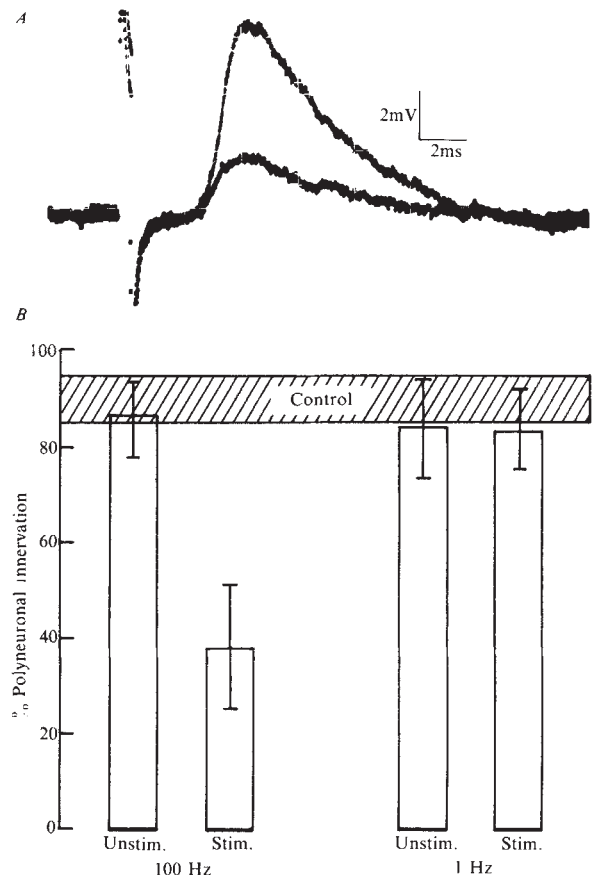


Fig. 1 A, Intracellular recording of endplate potentials from a muscle fibre showing the procedure used to determine whether individual muscle fibres were polyneuronally innervated. Shown are two superimposed oscilloscope sweeps, each at a different intensity of stimulation of the muscle nerve. The increase in the amplitude of the endplate potential at the higher intensity of stimulation shows that this fibre was innervated by at least two axons having slightly different thresholds to the stimulation of the muscle nerve. B, Histograms of percentage of muscle fibres exhibiting polyneuronal innervation in 10-11-day-old muscles following various procedures. The hatched portion of the histogram indicates the level (mean $\pm$ s.e.) of polyneuronal innervation present in 11 muscles of normal, untreated animals reared by their mothers. The pair of bars to the left represent the polyneuronal innervation in right soleus muscles stimulated with the 100 Hz pattern from day 7 (Stim.) and in the left, contralateral muscles (Unstim.) from 8 animals. The pair of bars to the right show the polyneuronal innervation present in muscles from 3 animals whose right soleus muscles received the 1 Hz stimulation pattern from day 7 (Stim.) and whose left soleus muscles served as controls (Unstim.). At least 20 fibres were sampled by intracellular recording in each muscle. Bars indicate s.e.m.

Synapse elimination in rat soleus muscle occurs during the first 2 weeks after birth⁶. Up to days 9-10, all of the fibres are innervated by more than one motor axon. At this age, synapse elimination begins to result in the appearance of singly innervated fibres, and by days 15-16 almost all of the fibres are singly innervated. To investigate the effects of chronic stimulation on this time course of synapse elimination, Teflon-coated multistranded stainless steel wires were drawn underneath the skin of 7-day-old rat pups anaesthetized with ether. The bared tips of these wires were implanted into the muscles anterior and posterior to the soleus in the right leg. Stimulus pulses of 1.0 ms duration and 4-6 mA were passed between the wires to activate the soleus muscle; these pulses were at least three times the intensity necessary to promote maximal plantar flexion. Stimuli were presented chronically to the pups for the next 3 to 4 days. To assure the animals suffered no pain from the stimuli, their spinal cords were transected at T11. In agreement with previous findings¹², cord transection in these

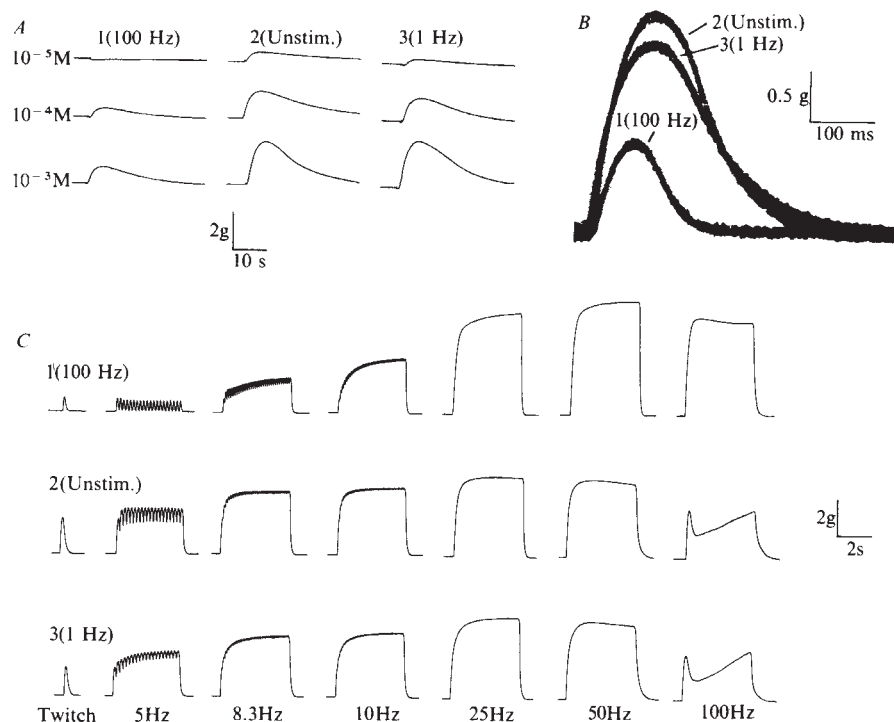


Fig. 2 Acetylcholine sensitivity and contractile properties of stimulated muscles. **A**, Muscle contractions to bath application of three concentrations of acetylcholine chloride, **B**, *in vitro* isometric twitches, and **C**, *in vitro* fusion of twitches at various frequencies of nerve stimulation. **A1**, **B1** and **C1** are results from the right soleus of an 11-day-old animal which had been stimulated with the 100 Hz pattern since day 7. **A2**, **B2** and **C2** are from the unstimulated left soleus muscle of the same animal as **A1**, **B1** and **C1**. **A3**, **B3** and **C3** are from the right soleus of a 12-day-old animal which had received stimulation continuously at 1 Hz since day 7.

neonatal animals did not noticeably alter the animal's reflexive and spontaneous use of his hindlimbs, nor was cord transection found to alter the normal time course of synapse elimination. Lastly, pups were maintained away from their mothers using an artificial rearing technique developed by Hall^{13,14}. This involved placing the pups in Styrofoam cups on a heated water bath and feeding them an artificial diet delivered from a syringe pump via an intraoral cannula. Pups maintained in this manner grew in excess of 1 g per day and underwent synapse elimination with the normal time course. At the end of 3–4 days of stimulation, both the right, stimulated and the left, unstimulated soleus muscles were dissected and their innervation and contractile properties examined at room temperature in a bath perfused with oxygenated mammalian Ringer's^{6,15}. The status of innervation of a sample of at least 20 muscle fibres in each muscle was determined by intracellular recording of endplate potentials in the presence of curare. The stimulus to the muscle nerve was carefully graded in intensity and the number of different threshold components to the endplate potential determined. If a fibre had two or more such components, as in Fig. 1A, it was considered polyneuronally innervated; fibres where only a single threshold component could be seen were considered singly innervated.

The effects of 3–4 days of chronic stimulation were found to be dependent upon the pattern of stimulation. Two patterns of stimuli were given to the muscles; the choice of these patterns was based upon previous studies showing that direct stimulation of denervated, adult muscles reduces the denervation hypersensitivity to acetylcholine in a pattern dependent fashion¹⁶. Each of the two patterns contained the same number of stimuli, an average of 1 stimulus per s. One pattern was a 100 Hz train lasting for 1 s presented every 100 s; this pattern is usually said to mimic the activity patterns of so-called 'fast' motor neurones and is very effective in reducing denervation hypersensitivity. The other pattern was a 1 Hz stimulation presented continuously; this pattern is more typical of so-called 'slow' motor neurones and is far less effective in reducing denervation hypersensitivity.

The polyneural innervation of the left, unstimulated muscles from both groups of stimulated animals was roughly the same as that found in untreated control animals: about 85% of the fibres in each muscle at 10–11 days of age received

innervation from more than one motor neurone (Fig. 2B). Stimulation of muscles with the 1 Hz pattern produced no alteration in this level of polyneural innervation. On the other hand, muscles receiving the 100 Hz pattern consistently had less than half this level of polyneural innervation. The most dramatic result was in one 11-day-old animal which had no polyneural innervation in the right, stimulated muscle whereas 75% of the fibres in the left, unstimulated muscle were polyneuronally innervated. The polyneural innervation found in the 100 Hz stimulated muscles was that which would be expected 2–3 days later in development. Stimulation with the 100 Hz pattern therefore produced a 2–3 day acceleration in the rate of synapse loss.

This acceleration in synapse loss produced by the 100 Hz pattern was not due to any change in the number of motor neurones to these muscles. Estimates of the numbers of motor units in such muscles made by counting increments in the twitch tension to graded nerve stimulation were the same as those in the contralateral, unstimulated muscles or in control muscles: four stimulated muscles had 17–23 increments compared with 18–22 increments in the contralateral muscles. Furthermore, cross-sections of the soleus nerve showed approximately the same number of large, myelinated axons in control and stimulated muscles (99–127 and 100–116 axons, respectively). Therefore, the 100 Hz stimulation pattern affects synapse elimination by reducing the number of synapses made by each of the soleus motor neurones.

As neonatal muscle fibres are supersensitive to acetylcholine¹⁷, an assessment was also made of the ability of these two stimulus patterns to alter the sensitivity of these fibres. Sensitivity was assessed by measuring the isometric contractures of each muscle to various concentrations of bath applied acetylcholine chloride. Each concentration of acetylcholine produced a smaller contracture in the 100 Hz stimulated muscles than in either the contralateral, unstimulated muscles or in the muscles stimulated at 1 Hz (Fig. 2A). Thus, the 100 Hz pattern, but not the 1 Hz pattern, produced a decrease in acetylcholine sensitivity.

Stimulation of muscles with the 100 Hz pattern also altered the muscles' contractile properties. Isometric twitches in these muscles had much more rapid rise times and smaller amplitudes than those of unstimulated or 1 Hz stimulated muscles (Fig.

2B). Despite their smaller twitch amplitudes, muscles receiving the 100 Hz stimulation consistently had larger tetanus contractions (Fig. 2C). Moreover, fusion of the twitches in these muscles required a higher frequency of stimulation of the muscle nerve. These same contractile properties were observed if the muscles were tested in these acute experiments by direct activation of the muscle fibres themselves; therefore, the altered contractions are due to changes in the fibres themselves rather than to changes in neuromuscular transmission. Changes in contractile properties similar to these have been observed in adult rat soleus muscles after stimulation at 100 Hz¹⁸ and are probably due to stimulus-induced changes in the duration of the muscle active state¹⁹, that is, in the duration of time that filament sliding occurs. Stimulation is therefore capable of altering the contractions of neonatal muscles in a pattern-dependent fashion.

These results reaffirm the previous observations of O'Brien *et al.*¹¹ showing that increased use of a muscle and its neuromuscular junctions accelerates elimination of synapses during early postnatal development. The present study extends these results in showing that the effectiveness of use in producing this acceleration is dependent upon the patterning of the imposed activity. In contrast to the study of O'Brien, no stimulus-induced changes in the muscle contralateral to that receiving stimulation were seen, showing that the stimulation is not causing any systemic changes, perhaps unrelated to increased muscle use. Additionally, the present experiments show that the acceleration of elimination is not explained by a stimulus induced destruction of a portion of the muscle innervation. Stimulated muscles having many fewer polyneuronally innervated fibres nonetheless had no fewer motor units than the contralateral, unstimulated muscles.

With the large stimulus currents used in this study, it is possible that intramuscular motor axons as well as the muscle fibres were stimulated. Therefore, because nerve fibres, neuromuscular junctions and muscle fibres were possibly all activated by the stimulation, it is not yet possible to determine at which of these sites the activity effect on synapse elimination is exerted. However, the parallel changes in innervation, muscle contractions and acetylcholine sensitivity indicate that all three may be controlled by common, activity-influenced mechanisms. It is already established that muscle activity exerts its effects on acetylcholine sensitivity and contractile properties in denervated, adult muscle by controlling the synthesis of proteins within the muscle fibre²⁰⁻²². By analogy, a possible activity effect on synapse elimination could be an influence on the production of a hypothetical 'innervation factor'^{23,24}, which is synthesized by the muscle fibres and which is an object of competition between the several innervating axons.

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A neuronal clone derived from a Rous sarcoma virus-transformed quail embryo neuroretina established culture

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Neuroretina (NR) is an evagination of the central nervous system (CNS) which is composed of photoreceptors, glial (Müller) cells and horizontal, bipolar, amacrine and ganglion neuronal cells. We describe here the usefulness of Rous sarcoma virus (RSV) in the establishment of a neuronal clone from quail embryo neuroretina. When primary cultures of chick and quail embryo neuroretina cells are transformed by RSV, neuronal markers such as ribbon synapses, choline acetyltransferase (CAT) and glutamic acid decarboxylase (GAD) specific activity are present¹. These RSV-transformed primary cultures can be established into permanent cell lines from which neuronal clones have been isolated. One of them, clone QNR/D, can generate tetrodotoxin (TTX)-inhibitable action potentials on electrical stimulation, has a high GAD activity and binds monoclonal antibodies raised against chick embryo neuroretina. The presence of these neuronal markers suggests that the QNR/D clone is derived from cells of the amacrine or ganglionic lineage. This is the first time that a neuronal cell clone of defined origin has been obtained from the CNS. The neuronal markers of the QNR/D clone are expressed at both the permissive and the non-permissive temperatures for transformation.

Neuroretinas were dissected from 7-day quail embryos, dissociated into essentially single cell suspensions and resuspended in Eagle's basal medium (BME) + 5% fetal bovine serum (FBS). About 10⁷ cells in a volume of 5 ml of BME + 5% FBS, mixed with one focus-forming unit of RSV, were plated into 25-cm² tissue culture flasks, as described previously¹. The RSV used here was ts NY-68, a transformation-defective thermosensitive mutant of RSV². The non-permissive temperature of this virus in chick embryo cells is 42 °C. As quail NR cells cannot be maintained in long-term cultures at temperatures exceeding 39 °C, they were kept between 38.5 and 39 °C. The progression of the RSV-transformed cultures to the permanently established cell line was a multi-stage process. The cells were first subcultured after 4 weeks and then a further three or four times. At this stage, about 4 months after the start of the culture, the cells stopped growing. This 'crisis' lasted a few weeks; once it was over, cell growth resumed and has not stopped since. Subculturing primary cultures of normal uninfected NR cells failed to produce any cell lines. The permanently established QNR ts NY-68 cell line was kept at 38.5-39 °C; very few cells were morphologically transformed at this temperature and cell loss and selection were therefore minimal. A shift to the transformation-permissive temperature of 36 °C resulted in a marked increase in the proportion of round cells. Cells synthesized and continuously released RSV in both temperature conditions (data not shown).

The QNR ts NY-68 cell line contained cells with neuronal properties. About 30-40% of the cells bound tetanus toxin, a marker of neuronal cells^{3,4} or the monoclonal antibody A2B5, which seems to be specific for neurones in avian embryo neuroretina cultures^{1,5}. Although no CAT activity was detected, GAD specific activity⁶ was always present, at levels as high as 60 nmol CO₂ per h per mg protein. Electrophysiological studies

showed an average membrane potential of -37 mV. During the first 18 months in culture only minor signs of membrane excitability (delayed rectification and weak partial response) were seen. Thereafter, 10–20% of the cells in which stable penetration was obtained showed typical action potentials (data not shown). The reasons why excitable cells could only be detected 1 yr after the establishment of the cell line are unclear. One possibility was that they represented an enrichment of this particular cell type; conversely, these cells might have been present from the very start of the culture, but the expression of their excitability required the elimination of another cell type. Whatever the explanation, membrane excitability could either be a permanent property of the cells or else only be expressed in specific conditions.

To resolve these problems, the permanently established mixed cell culture was cloned. The method used was dilution cloning according to the Poisson distribution. Suspensions obtained by a brief treatment with a trypsin (Gibco, cat. no. 509)-containing 1:5,000 versene solution (Gibco, cat. no. 043-5040) were composed of more than 95% of single cells which were plated into 96-well Costar plates in BME + 20% FBS. As preliminary experiments had shown that clones could not be obtained without feeder layers, the cell suspension to be cloned was mixed with a suspension of the same cells which had been lethally irradiated. Five clones were obtained in this manner, one of which, QNR/D, has been thoroughly studied. The cells were routinely maintained at 38.5 – 39°C , but their properties were also studied at 36°C , the permissive temperature for transformation. At 39°C and after each subculture, most cells of the QNR/D clone were flat with large somas; their morphology changed gradually (Fig. 1) and 7 days later almost all cells had small somas with two or more long thin processes. About 50% of the cells, whatever their morphology, strongly bound tetanus toxin or A2B5 at all stages of the culture (data not shown). The situation is similar to that reported in brain primary cultures where neurones are not always stained equally well by tetanus toxin^{7,8}.

The electrophysiological properties were studied in cells with stable cellular recordings. All the cells of clone D which have been tested produced action potentials when stimulated with brief depolarizing currents (Fig. 2). The action potentials had a slow rising phase ($<20\text{ V s}^{-1}$) and did not overshoot the zero membrane potential. The bath application of TTX⁹ at a concentration of $0.1\text{ }\mu\text{M}$ blocked the action potential in less than 5 min and no action potentials were seen in sodium-free medium. Although large cells were easier to penetrate than small ones, action potentials were recorded from both sizes of cells, whether isolated or in clusters. These electrophysiological properties seem to be stable because they have been found repeatedly during the course of 50 cell doublings as well as in cells which were thawed after being frozen in liquid nitrogen.

The amacrine and ganglion cells are probably the NR neurones which generate action potentials *in vivo*¹⁰. We have therefore searched for other markers which might indicate that clone D is derived from one of these cell types. We selected to study the synthesis of putative neurotransmitters and the binding of monoclonal antibodies specific for the avian retina and/or CNS. The GAD-specific activity was 40–80 nmol per h per mg protein and preliminary data on the free amino acids present showed that clone D contained ~ 2 nmol of GABA and ~ 50 nmol of taurine per mg protein (D. Cambier, personal communication). Twenty monoclonal antibodies raised against chick embryo neuroretina (provided by G. D. Trisler and M. Nirenberg) were also screened on the excitable QNR/D clone. The surface of cells was labelled by 10 different antibodies including 14H3 (ref. 11) and an antibody (94C2) that seems to bind to amacrine and ganglion cells in sections of chick embryo neuroretina (G. D. Trisler, personal communication) (Fig. 3). Nearly all cells were labelled by 14H3 and $\sim 60\%$ by 94C2. Taken together, the electrophysiological, biochemical and immunological data suggest that clone D may be the *in vitro* counterpart of the amacrine or ganglion cells. All differentiation

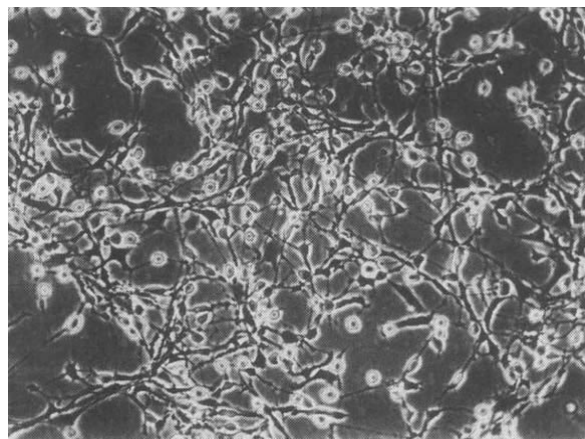


Fig. 1 Phase contrast micrograph of neuroretina clone QNR/D cells 4 days after subculture. The majority of cells have small somas with long processes and the others are flat with large somas. $\times 90$.

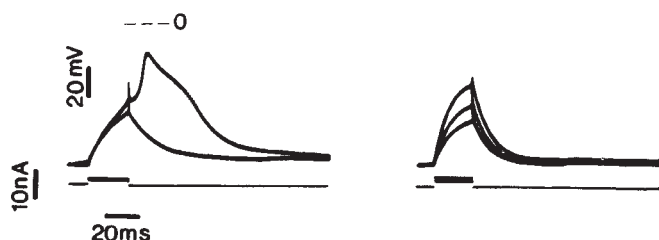


Fig. 2 Response of cells from the QNR/D clone to electrical stimulation. Cells dissociated by mechanical dissociation were plated into 35-mm diameter Falcon tissue culture dishes in BME + 5% FBS and kept at 36 or 39°C in an atmosphere of 95% air, 5% CO_2 . Electrophysiological recordings were usually made 4 days after the subculture, 24 h after a medium change. The culture medium was replaced by a solution containing 115 mM NaCl, 5.4 mM KCl, 0.4 mM MgCl_2 , 1.8 mM CaCl_2 , 25 mM HEPES, 5 mM glucose and buffered at pH 7.4. The culture dish was placed on the warm stage of an inverted microscope (Leitz Diavert) and the temperature maintained at $36 \pm 1^\circ\text{C}$. Glass capillary microelectrodes were used for intracellular recording and stimulation. They were filled with equal volumes of 3 M KCl and 4 M potassium acetate and had resistance values in the range 50–80 $\text{M}\Omega$. The reference electrode was connected to a negative capacitance electrometer amplifier (WP 1707) with an active bridge network which allowed the simultaneous injection of current and voltage recording through the same microelectrode. The resting potential ranged between -40 and -60 mV. The membrane potential was adjusted to a steady level from -70 to 80 mV by passing a constant current. Left, action potentials evoked by brief depolarizing currents; right, after a bath application of $0.1\text{ }\mu\text{M}$ TTX. Upper trace, zero voltage line; lower trace, current trace.

markers were expressed in cells maintained at 36°C as well as at 39°C . For example, electrophysiological experiments were done on cells maintained at 37°C for at least 3 days and in some experiments for as long as 2 months, that is, in conditions in which many cells are morphologically transformed. The expression of transformation parameters therefore did not interfere with the electrophysiological properties.

Whether or not NR cells transform into permanent lines depends on the species used. Indeed, despite the fact that chick NR can be induced to proliferate¹², all our attempts to derive permanent cell lines from chicken NR cells have been unsuccessful. This contrasts with the situation in the quail where independent cultures, including the one from which the QNR/D clone was derived, have been obtained by transformation with *ts* NY-68. Even in the quail, transformation by RSV is a prerequisite for the establishment of permanent cell lines; in uninfected controls, neurones disappear at the first subculture and no neuronal cell line can therefore be established.

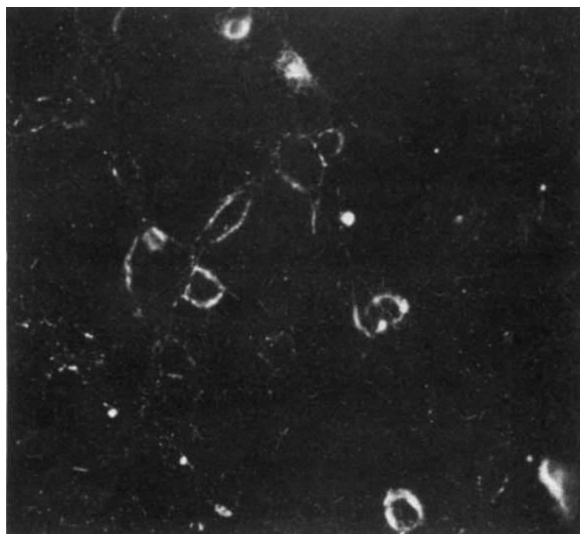


Fig. 3 Visualization of binding of the monoclonal antibody 94C2 to QNR/D cells by indirect immunofluorescence. Cells grown on glass coverslips were washed three times with Hanks' solution containing 0.01 M HEPES and incubated with the undiluted culture medium of 94C2 hybridoma cells for 120 min. After washing in Hanks', cells were incubated with a 1:20 solution of fluorescein-conjugated goat anti-mouse immunoglobulin (Nordic) for 30 min. All incubations were at 4 °C. Cells were washed, fixed overnight in a 3% solution of buffered formaldehyde, mounted on slides in buffered glycerol and viewed with an epifluorescence Leitz microscope with appropriate filters. $\times 540$.

Various authors have shown that the process of differentiation seems to be inhibited when primary cultures of differentiating chick embryo cells are infected with a *ts*, transformation-defective, mutant of RSV, and kept at the permissive temperature. These mutants inhibit the synthesis of proteoglycan by chondrocytes¹³, prevent the fusion of myoblasts and the synthesis of specific muscle biochemical markers¹⁴ and block the synthesis of melanin¹⁵ in retina melanoblasts. In contrast, in primary cultures of chick or quail embryo neuroretina cultures, transformed by *ts* NY-68 at 36 °C, neuronal cells retain or acquire differentiation markers such as synapses or the synthesis of neurotransmitters¹ and the neuronal cell clone described here displays many differentiated properties. The interaction of RSV with cultured cells of the nervous system therefore seems to differ from the one occurring with other cell types. The methodological approach used here may also be relevant to other regions of the quail nervous system and enable additional neuronal excitable cell lines to be derived. Those available at present include the neuroblastoma¹⁶ and the pheochromocytoma cell lines¹⁷ and the quail embryo neuroretina clone described here.

Follow-up studies on the biochemical, pharmacological and electrophysiological properties of the QNR/D clone may help to define the characteristics of the cells from which they are probably derived, that is, amacrine or ganglion cells, and their interactions with other retina cell types and with cells of the optic tectum.

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Novel form of drug-dependence—on adenosine in guinea pig ileum

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There is evidence that dependence on opiates occurs in neurones bearing their specific receptors, whose activation inhibits the neurone¹. Thus, incubation of guinea pig ileum with an opiate induces in the final cholinergic motoneurone of the myenteric plexus a dependence that closely resembles in basic characteristics opiate dependence in whole animals^{2–6}. A comparable, but distinct dependence can be induced by incubating the ileum with clonidine⁷. Since adenosine also inhibits the final cholinergic motoneurone, by activating a specific purine receptor^{8,9}, we have tested whether it, too, can induce a distinct dependence in this neurone. To demonstrate dependence, we challenged the ileum by removing drug or by adding the selective purine receptor antagonist, 8-phenyltheophylline, which does not substantially inhibit phosphodiesterase, or caffeine^{10–13}. We found that incubation of the ileum with adenosine, or with the more potent derivative, 2-chloroadenosine^{14–16}, induced a novel form of drug dependence, made manifest by withdrawal of inducing drug, but not by antagonists of opiates or clonidine.

Administered acutely to fresh preparations of guinea pig isolated ileum, adenosine and its analogues inhibit the release of acetylcholine from the terminal of the motoneurone, and hence inhibit contraction of the longitudinal muscle, evoked by electrical stimulation^{8,17–22}. Using preparations set up in Krebs solution containing 70 μ M hexamethonium, we obtained the following mean values for the concentration required to produce 50% inhibition of the electrically-evoked contraction (acute IC_{50}): adenosine, 1.6 μ M $\pm$ 0.2 s.e.m. (n = 13); 2-chloroadenosine, 0.069 μ M $\pm$ 0.011 (n = 6). Both 8-phenyltheophylline and caffeine antagonized the action of adenosine and 2-chloroadenosine. 8-Phenyltheophylline was about 50 times more potent than caffeine.

In experiments on dependence induction, the essential procedure resembled that already used in inducing dependence on normorphine or clonidine in the ileum *in vitro*^{6,7}. Pieces of ileum were preincubated for 16–21 h at 18–22 °C in Krebs solution containing 70 μ M hexamethonium (the incubation fluid) with or without added drug(s). After preincubation, the preparation was set up in fluid of the same composition at 37 °C for recording the contracture of the longitudinal muscle. During 30–40 min at 37 °C, the preparation was challenged with acetylcholine, to determine the maximal response to this transmitter, and with short series of electrical stimuli, to establish that the neurones were reactive. Usually, test and control preparations, derived from the same ileum, were treated and set up in parallel. Test and control preparations showed parallel sensitivities to acetylcholine.

Fresh preparations of ileum, without preincubation, set up at 37 °C in incubation fluid containing adenosine and challenged 30–40 min later with 8-phenyltheophylline, gave little or no contracture (Table 1a). Similarly, preparations preincubated without test drug and then set up at 37 °C, gave little or no response to 8-phenyltheophylline and a small response to caffeine (Table 1b, c). After preincubation with adenosine or

Fig. 1 Response of adenosine-dependent preparations of guinea pig isolated ileum to replacement of incubation fluid containing adenosine by fluid without adenosine; and suppression by adenosine of the 'spontaneous' withdrawal response. Two preparations derived from the same ileum, after preincubation in Krebs solution containing $4 \mu\text{M}$ adenosine, were set up in separate organ baths to record contracture of the longitudinal muscle in parallel. Adenosine ($4 \mu\text{M}$) was present in the bath fluid throughout the experiment, except for the periods indicated by hatched horizontal bars above the tracing. Reference responses to electrical stimulation (ES) and to acetylcholine (ACh) are also shown. Further details are given in Table 1 legend.

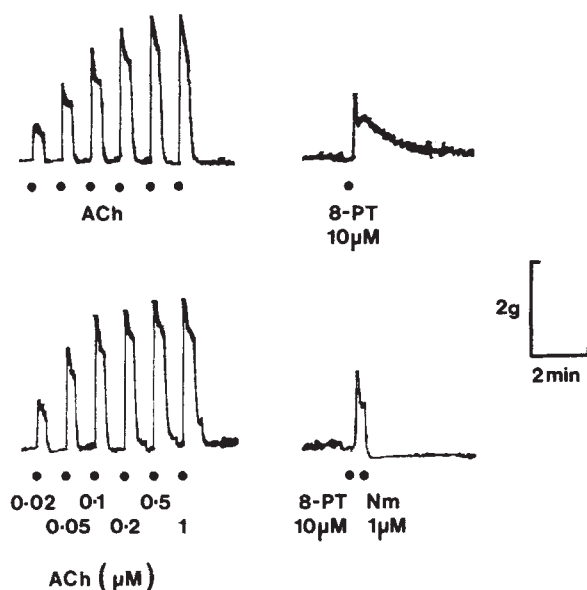
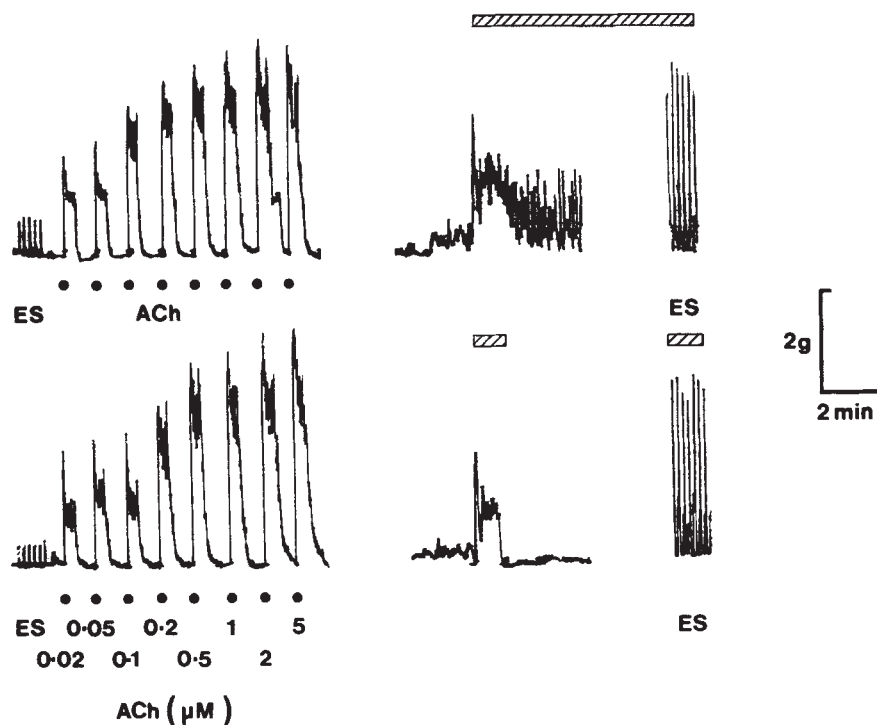


Fig. 2 Suppression by $1 \mu\text{M}$ normorphine (Nm) of withdrawal contracture precipitated with $10 \mu\text{M}$ 8-phenyltheophylline (8-PT) in adenosine-dependent preparation. Adenosine ($4 \mu\text{M}$) was present in the bath fluid throughout the experiment. Other details as in Table 1 and Fig. 1.

2-chloroadenosine at $18-22^\circ\text{C}$, the preparation responded with a withdrawal contracture to challenge with 8-phenyltheophylline (Table 1d, e, f, j) or caffeine (Table 1g). The tension of this contracture was directly related to the concentration of adenosine (Table 1d, f) and to the challenge dose of 8-phenyltheophylline (Table 1e, f). When 8-phenyltheophylline was included with adenosine during the whole period of preincubation, responsiveness to challenge was reduced (Table 1h).

Figure 1 shows that, after setting up at 37°C , removal of adenosine from a preparation preincubated with it elicited a spontaneous withdrawal contracture that could be suppressed by fresh addition of adenosine. This experiment and those summarized in Table 1 indicate that preincubation of pieces of ileum with adenosine or 2-chloroadenosine, followed by setting up at 37°C in incubation fluid of the same composition, induces a state of adenosine dependence, expressed as a contracture of the longitudinal muscle on withdrawal of inducing drug.

Table 1 Withdrawal contractures of guinea pig ileum after preincubation in various fluids *in vitro*

Addition to incubation fluid	n	Challenge	Mean response $\pm$ s.e.m.	P value versus
(a) None, not preincubated	13	$10 \mu\text{M}$ 8-PT	13.1 ± 5.3	(f) <0.001
(b) None	7	$10 \mu\text{M}$ 8-PT	8.6 ± 4.4	(f) <0.001
(c) None	7	1 mM Caffeine	24.4 ± 3.1	(g) <0.02
(d) $1 \mu\text{M}$ Ao	7	$10 \mu\text{M}$ 8-PT	24.6 ± 4.6	(f) <0.001
(e) $4 \mu\text{M}$ Ao	10	$2.5 \mu\text{M}$ 8-PT	40.3 ± 7.4	(f) <0.02
(f) $4 \mu\text{M}$ Ao	10	$10 \mu\text{M}$ 8-PT	67.1 ± 6.4	—
(g) $4 \mu\text{M}$ Ao	7	1 mM Caffeine	46.9 ± 7.3	—
(h) $4 \mu\text{M}$ Ao + $1 \mu\text{M}$ 8-PT	7	$10 \mu\text{M}$ 8-PT	19.8 ± 6.1	(f) <0.001
(j) 60 nM 2-CA	11	$10 \mu\text{M}$ 8-PT	31.5 ± 4.8	(b) <0.01
(k) $4 \mu\text{M}$ Ao	5	$0.1 \mu\text{M}$ Nx	ND	—
(l) $4 \mu\text{M}$ Ao	4	$1 \mu\text{M}$ Yo	ND	—

Pieces of ileum were preincubated for 16–21 h at $18-22^\circ\text{C}$ in Krebs solution (mM: NaCl, 119; KCl, 4.7; CaCl_2 , 2.5; MgSO_4 , 1.2; KH_2PO_4 , 1.2; NaHCO_3 , 25; glucose, 11) containing $70 \mu\text{M}$ hexamethonium gassed with 95% O_2 and 5% CO_2 , with or without addition of test drug. The incubation fluid was arranged to flow over the ileal pieces at 2.5 ml per min . Additions to the incubation fluid were adenosine (Ao), 8-phenyltheophylline (8-PT) or 2-chloroadenosine (2-CA). After preincubation, preparations were set up in incubation fluid of the same composition at 37°C for recording with a Harvard isometric transducer the contraction of the longitudinal muscle in response to transmural stimulation with square-wave electrical pulses at 0.1 Hz and supramaximal voltage. Preparations were challenged by addition to the bath of the selective purinoceptor antagonists, 8-PT or caffeine, or of naloxone (Nx) or yohimbine (Yo). Responses were measured as a percentage of the maximal response of the preparation to acetylcholine. The significance of differences between means was determined with Student's *t*-test. ND, not detectable.

To distinguish adenosine dependence from that previously demonstrated on opiates and clonidine, we challenged the adenosine-dependent preparation with naloxone or yohimbine, respectively antagonists of opiates and clonidine. Neither drug elicited a withdrawal contracture, although 8-phenyltheophylline readily did so (Table 1k, l). Despite the failure of naloxone or yohimbine to elicit a withdrawal contracture from adenosine-dependent preparations, both normorphine (Fig. 2), acting on

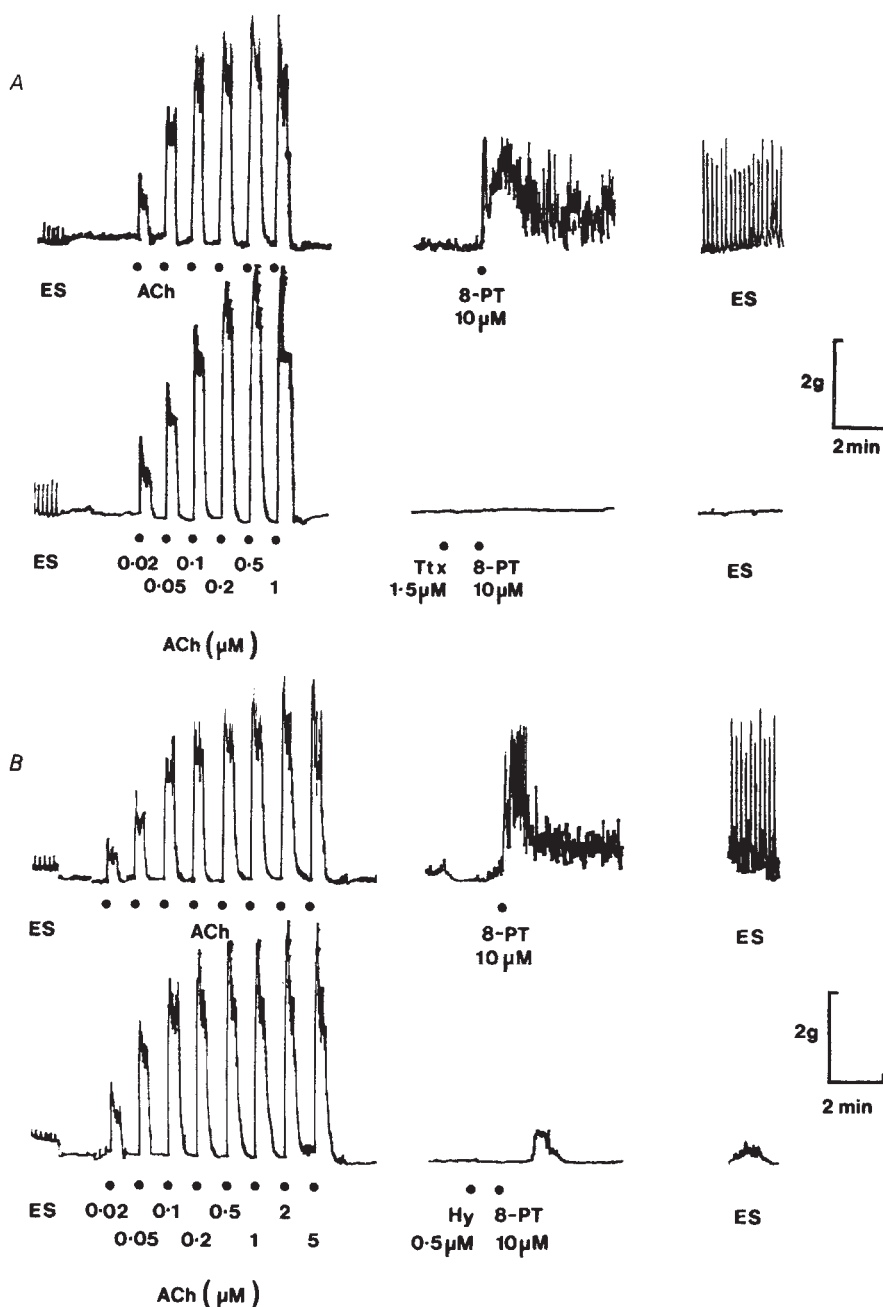


Fig. 3 Suppression of withdrawal contracture precipitated with 10 μ M 8-phenyltheophylline (8-PT) in adenosine-dependent preparations. **A**, Complete suppression by 1.5 μ M tetrodotoxin (Ttx); **B**, incomplete suppression by 0.5 μ M hyoscine (Hy). Details as in Table 1, Figs 1, 2.

the μ -receptor for opiates, and noradrenaline (data not shown), acting on the α -adrenoreceptor, suppressed the withdrawal contracture to 8-phenyltheophylline. Thus, although the recognition sites involved in dependence on adenosine, opiates and clonidine in the final cholinergic motoneurone are distinct, the final common path for the expression of withdrawal appears to be the same for all three categories of drug. These three dependences may therefore be described as 'convergent'.

Tetrodotoxin fully suppressed the withdrawal contracture, whether spontaneous or precipitated (Fig. 3A), indicating that adenosine dependence occurs in neurones of the myenteric plexus. Over a 6-month period of experiments, the inhibitory effects of hyoscine on the withdrawal contracture varied from almost complete suppression (Fig. 3B) to no more than a retardation of the onset of the response. This suggests that not only acetylcholine, but also another transmitter contributes to the withdrawal effect, as previously proposed for opiate withdrawal in the ileum^{23,24}. Since all experiments were done in the presence of hexamethonium, our observations with hyoscine implicate the final cholinergic motoneurone as a site of adenosine dependence, which is consistent with the suppressive effects of normorphine and noradrenaline on the adenosine

withdrawal contracture. These observations also indicate a need to investigate the possibility that a second transmitter is also involved.

Dependence on adenosine or 2-chloroadenosine adds, to those on opiates²⁻⁶ and clonidine⁷, a third form of dependence on an inhibitory drug that can be induced in the final cholinergic motoneurone *in vitro*, and provides another tool for the analysis of dependence mechanisms. Adenosine dependence also adds to that of [Met]enkephalin and β -endorphin^{5,25} an example of the induction of dependence by an endogenous substance, albeit from an exogenous source. Since the purine receptor involved in adenosine dependence in the ileum is probably of the subtype termed A1, or Ri (refs 9, 15, 26, 27), the activation of which inhibits adenylate cyclase in situations where this has been tested, the above observations are consistent with the hypothesis that dependence can occur, after initial inhibition by drug of adenylate cyclase, through a compensating hypertrophy of the cyclic AMP generating system^{1,28}.

Hitherto, caffeine has been thought to induce arousal by the acute antagonism of the inhibitory effect of adenosine in the brain^{29,30}. A further reinforcing factor may also apply. The finding that adenosine, which is generated endogenously in the

brain²⁷, can induce dependence and that caffeine can precipitate abstinence suggests that part of the arousing effect of caffeine might be due to its precipitating withdrawal in neurones of the brain that have become dependent on adenosine, if its presence is continued for long enough.

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Role of *de novo* DNA methylation in the glucocorticoid resistance of a T-lymphoid cell line

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A correlation has been shown between changes in the methylation pattern of cytosine residues in DNA and the expression of specific genes in differentiated tissues¹⁻⁶. The pattern of DNA methylation is conserved, through cell division, by a maintenance methylase⁷⁻⁹ but the mechanism by which a given pattern of methylation is established is unknown. *De novo* methylation of foreign DNA molecules has been shown to occur in several systems¹⁰⁻¹², and may serve as a signal to arrest gene expression. Conversely, treatment of cultured cell lines with 5-azacytidine results in DNA hypomethylation and leads to transcriptional activation of previously unexpressed genes¹³⁻¹⁸. The results described here demonstrate spontaneous *de novo* methylation of DNA in a T-lymphoid cell line previously treated with 5-azacytidine to generate glucocorticoid sensitivity. This *de novo* methylation is accompanied by the acquisition of the glucocorticoid-resistant phenotype.

The SAK8 cell line was derived from a spontaneous thymic lymphoma in an AKR mouse. Previous work has shown that SAK8 cells contain fully functional hormone receptors but are

resistant to cytolysis by glucocorticoid hormones due to the absence of a lysis function¹⁹. We have shown that treatment of SAK8 cells with 5-azacytidine generates subclones sensitive to dexamethasone, a synthetic glucocorticoid, at a frequency of about 10% (ref. 19). Thus, it appears that the origin of glucocorticoid resistance in SAK8 cells is methylation of a non-receptor gene(s) involved in the cytolytic response, possibly as a consequence of differentiation. This report describes our characterization of the dexamethasone-sensitive subclones generated by treatment of SAK8 cells with 5-azacytidine.

Independent dexamethasone-sensitive subclones were derived, in three separate experiments, from 5-azacytidine-treated SAK8 cells by subcloning at a dilution of 0.25 cells per well. Six independent clones were cultured in the absence of glucocorticoids and tested for continued hormone sensitivity after varying intervals. Five of the six subclones were found to revert to dexamethasone resistance over 1-2 months. The growth of one clone, SAK8.A2, in the presence of dexamethasone after 1, 4, 8 and 10 weeks in culture is shown in Fig. 1. There is a gradual shift in the phenotype of SAK8.A2 from dexamethasone-sensitive (week 1) to resistance (week 10); the growth of the cells after 10 weeks in culture is nearly identical to that of untreated SAK8 cells in the presence of dexamethasone. After 8 weeks in culture the SAK8.A2 cells were subcloned in the absence of hormone and screened for dexamethasone sensitivity. Of the 40 subclones examined, 13 were resistant, 10 remained sensitive and 17 showed partial resistance, indicating that the intermediate phenotypes seen (Fig. 1, weeks 4 and 8) represent mixed populations.

It seemed possible that this reversion process could be due to the loss of genetic material from an unstable population of cells. However, karyotypic analyses revealed no change in the

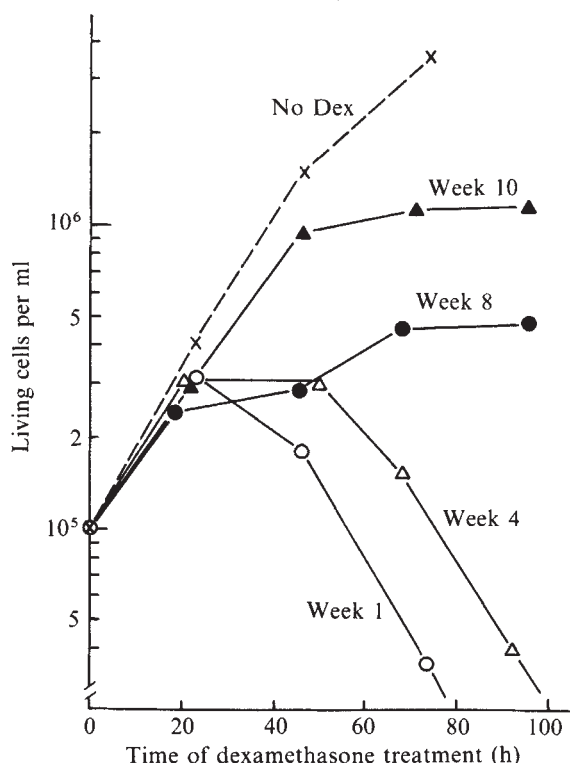
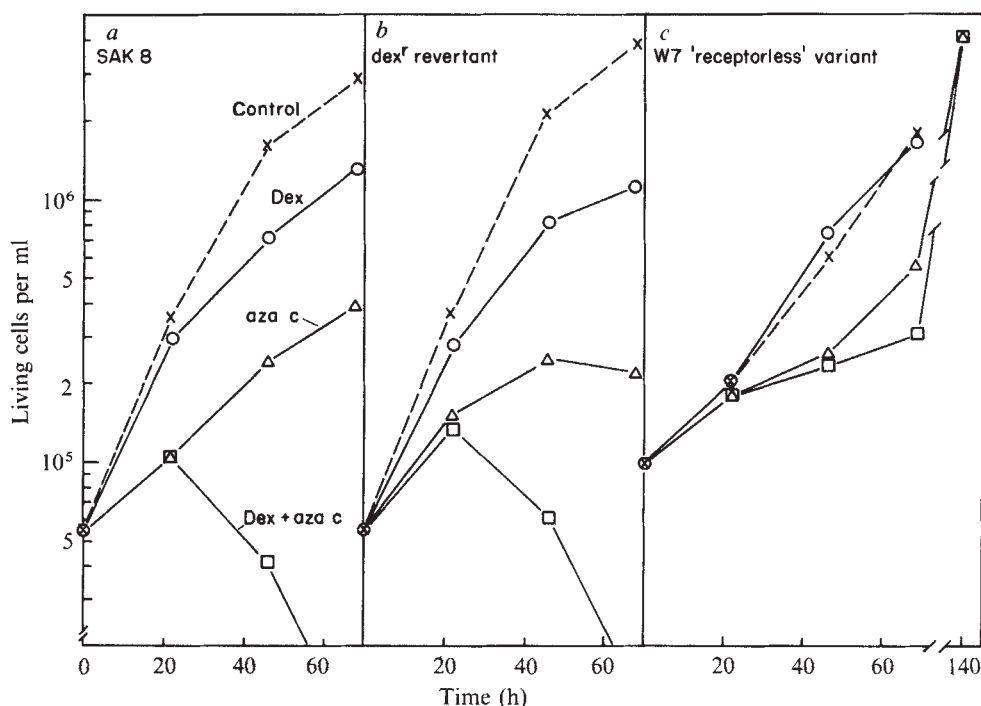


Fig. 1 Reversion of SAK8.A2 to the dexamethasone-resistant phenotype. SAK8 cells were treated for 13 h with 3 μ M 5-azacytidine, allowed to recover until exponential growth had resumed (48 h), subcloned and screened for dexamethasone sensitivity as described previously¹⁹. Clone SAK8.A2 was cultured in Dulbecco's modified Eagle's medium with 10% fetal calf serum and aliquots of cells were assayed for sensitivity to 10^{-6} M dexamethasone at weekly intervals. The growth of the cells in the absence of dexamethasone (---) did not change with time; however, sensitivity decreased with time in culture as shown. Living cells were monitored by trypan blue exclusion.

Fig. 2 Activation of glucocorticoid sensitivity by 5-azacytidine. SAK8 (a) and revertant SAK8.A2 cells obtained after 3 months in culture (b) were assayed in parallel for sensitivity to 1 μ M dexamethasone (Dex) $\circ$, 2 μ M 5-azacytidine (aza c) $\triangle$ and the combination of dexamethasone and 5-azacytidine ($\square$). Cells grown in the absence of dexamethasone and 5-azacytidine are shown for comparison (---). Living cells were determined by trypan blue exclusion. The slight increase in sensitivity of SAK8.A2 revertant cells to 5-azacytidine alone is reproducible and likely to be a result of the decreased levels of 5-methylcytosine in these cells. Both SAK8 and SAK8.A2 revertant cells are resistant to the lytic effects of dexamethasone, but the doubling time of both cell lines is increased in the presence of dexamethasone, as described elsewhere¹⁹. c, The receptorless variant is variant AN6 derived from the dexamethasone-sensitive W7 line²⁰. In that case a concentration of 0.1 μ M 5-azacytidine was used which, in W7 lines, results in a level of toxicity comparable to that of 2 μ M 5-azacytidine in the SAK8 line.



modal number of chromosomes. Analysis of cellular DNA content by flow microfluorimetry showed no differences among SAK8, dexamethasone-sensitive SAK8.A2 and dexamethasone-resistant SAK8.A2 revertants. ³H-dexamethasone binding assays confirmed the presence of the full amount of glucocorticoid receptor in the revertant cells (data not shown).

Because dexamethasone-sensitive SAK8.A2 cells were generated from SAK8 by hypomethylation of the DNA, we investigated the possibility that reversion could be due to spontaneous DNA remethylation. Instead of repeating the 13-h 5-azacytidine treatment followed by subcloning and screening of the clones, we developed a very simple assay which takes only 3–5 days. SAK8 and fully resistant SAK8.A2 (reverted to resistance during growth in culture for 3 months, see Fig. 1) were treated with dexamethasone and 5-azacytidine alone and in continuous combination. Figure 2a and b show that both glucocorticoid-resistant cell lines become glucocorticoid-sensitive, as they are lysed by dexamethasone in the continued presence of 5-azacytidine. These data support the idea that genes involved in the lysis response have been remethylated in the revertant line.

To demonstrate that the treatment with dexamethasone and 5-azacytidine together did not cause cytotoxicity through non-specific toxicity, we used a derivative of the dexamethasone-sensitive W7 cell line which had become resistant because of a mutation in the glucocorticoid receptor²⁰. In this case, there is no functional glucocorticoid receptor to mediate the lytic response and, as could be predicted, the cells are not lysed by dexamethasone even in the presence of 5-azacytidine (Fig. 2c). Two other cell types (B-9 glial cells and P-3 B cells) not originating from the T-cell lineage were also tested and found to remain resistant to glucocorticoid-induced cytotoxicity in the presence of 5-azacytidine. Glucocorticoid hormones alone increase the doubling times of both B-9 and P-3 cells, confirming the presence of functional receptor (data not shown).

To obtain biochemical evidence correlating the reversion to glucocorticoid resistance with *de novo* methylation, total 5-methylcytosine content of the DNA was measured using HPLC. Table 1 shows that in untreated SAK8 cells ~4.5% of the cytosine residues are methylated; this value is in good agreement with published values for mammalian DNA²¹. Following 5-azacytidine treatment and subcloning of the cells, 5-methylcytosine content is decreased by nearly 50% (2.5% of the total

Table 1 Quantitation of 5-methylcytosine content by HPLC

Cell line	$\frac{m^5dC}{m^5dC + dC} \times 100 \pm 1 \text{ s.d.}$
SAK8	4.5 ± 0.26
SAK8.A2 (weeks in culture)	
1	2.5 ± 0.09
4	3.1 ± 0.08
9	3.8 ± 0.22

Cellular DNA was prepared from SAK8 and at weekly intervals from SAK8.A2 by a modification of the method of Pellicer²⁸. The lysis buffer contained 10 mM PIPES (pH 6.8), 30 mM NaCl, 250 mM sucrose, 2 mM CaCl₂, 2 mM MgCl₂ and 0.4% NP40. Nuclei were pelleted through a cushion of equal volume of the above buffer without NP40 but 1 M in sucrose. The DNA samples were digested to completion with DNase I, venom phosphodiesterase and bacterial alkaline phosphatase²⁹. The nucleosides were separated using an Altex HPLC fitted with an ODS 256-05 reverse phase column, eluted with 0.05 M KH₂PO₄ (pH 4.5), containing 5.5% methanol at 2,500 p.s.i and 1.5 ml min⁻¹. The elution of the 1 μ g samples was monitored at 254 nm and the area under the peaks determined. Total base ratios were calculated from the integrator values, corrected for the appropriate extinction coefficients and are in agreement with published reports (data not shown). The mean (± 1 s.d.) is given for three analyses of each sample. Representative weeks in the reversion process are shown.

cytosines are methylated). Values are given for representative weeks during the reversion process showing a gradual increase in 5-methylcytosine, over time, from 2.5% to 3.8%, correlating with the acquisition of glucocorticoid resistance (Fig. 1). These results further support the hypothesis that glucocorticoid-sensitive derivatives of SAK8 revert to hormone resistance by spontaneous *de novo* methylation. As there are at least two alleles, and possibly more than one locus determining the lysis function, the partially resistant phenotypes observed in the mixed populations, shown in Fig. 1, could represent incomplete remethylation.

Several studies have characterized the activity of maintenance methylases^{7–9,22} (which preserve the existing pattern of DNA methylation) and of demethylating enzymes²³ which may be important in gene activation. *De novo* methylation has been postulated to have a role in differentiation^{24,25} and recently pre-implantation mouse embryos have been shown to have high levels of *de novo* methylase activity²⁶. To date, the effects of

de novo methylation on cellular genes in mammalian cells have been difficult to analyse. Previous reports of *de novo* methylation in mammalian cells have used the introduction of foreign DNA^{10,12,26} or have analysed DNA methylation at short times during recovery from 5-azacytidine treatment (0–48 h)²⁷. Our studies show *de novo* methylation of cellular DNA during the transition from glucocorticoid sensitivity to resistance; this *de novo* methylation must be an important step in the acquisition of glucocorticoid resistance because revertant cells again become dexamethasone-sensitive in the presence of 5-azacytidine, whereas receptor-defective variants of W7 do not. Note that demethylation alone is not sufficient to cause glucocorticoid sensitivity in other cell types tested (glial, B-cells) indicating other essential levels of control. It remains to be

determined whether the extensive demethylation caused by 5-azacytidine has activated *de novo* methylation or merely provided new sites for an existing activity. The use of 5-azacytidine to reversibly activate previously silent genes involved in cytolysis provides an interesting system in which to study the control of this differentiated T-cell function.

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Enhanced expression of H-2K and H-2D antigens on reticulocytes infected with *Plasmodium yoelii*

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The 17XNL strain of *Plasmodium yoelii* induces a highly effective and permanent T-cell dependent immunity in mice of the CBA strain^{1–3}; the lethal variant *P. yoelii* 17XL and *P. berghei* (ANKA) fail to activate an effective immune response in the same host^{2–4}. These differences in immunogenicity are unexplained. We recently observed that in CBA/CaJ mice the intracellular blood stages of *P. yoelii* 17XNL were almost exclusively within reticulocytes whereas lethal *P. yoelii* 17XL and *P. berghei* (ANKA), at comparable stages of infection, were predominantly erythrocytic. Induction of a reticulocytosis converted the normally lethal *P. yoelii* 17XL infection into a nonlethal one, and reticulocytic *P. yoelii* was shown to be more immunogenic than the erythrocytic form. Since one of the differences between reticulocytes and erythrocytes that might have influenced the development of immunity was greater expression of MHC antigens of the former cell type^{5–7} we examined the expression of H-2K, H-2D and Ia on reticulocytes infected with *P. yoelii* 17XNL. These cells showed a very marked increase in H-2K and D antigen expression compared to normal reticulocytes or erythrocytes. No Ia was detected. Red blood cells (RBC) infected with lethal *P. yoelii* 17XL or *P. berghei* showed no increase in H-2K or H-2D antigen expression. Finally, the level of expression of H-2K on *P. yoelii* 17XNL parasitized red blood cells from different strains of mice correlated closely with the ability of these strains to control the infection.

In CBA/CaJ mice, nonlethal *P. yoelii* 17XNL is found almost exclusively in immature RBCs (reticulocytes) while the lethal variant is predominantly in mature erythrocytes (Fig. 1a). To determine whether there is a more general relationship

between the age of the red cell parasitized and the severity of malarial infections, we examined *P. vinckei*, *P. berghei* (ANKA) and *P. chabaudi* infections in CBA mice (Table 1). *P. vinckei*, which killed CBA mice in 7–8 days, was restricted to erythrocytes; *P. berghei* (ANKA), which proved fatal in 20–25 days, was predominantly in erythrocytes in the first 10–14 days of infection but invaded reticulocytes later; *P. chabaudi*, which proved fatal in 30% of mice, was found in mature erythrocytes (predominantly), as well as in reticulocytes. It is also worth noting that *P. falciparum*, which causes the most severe form of human malaria, is found mainly in mature erythrocytes^{8,9}; *P. vivax* and *P. ovale*, which cause mild, non-fatal infections, are found predominantly in reticulocytes^{10–12} and *P. vivax* is known to induce good immunity¹⁰.

As these observations suggested that reticulocytic forms of malaria may be controlled better than erythrocytic forms we examined the effect of inducing a reticulocytosis (with phenylhydrazine) soon after infection with lethal *P. yoelii* 17XL (Fig. 1b). In phenylhydrazine-treated mice, parasites were found to invade reticulocytes and all treated mice recovered by day 18. Stimulation of reticulocytosis also protected mice against *P. vinckei*¹³ and potentiates resistance to *P. chabaudi*¹⁴. To compare more directly the immunogenicity of the reticulocytic and erythrocytic forms of *P. yoelii* we examined the ability of formalin-fixed parasitized RBC (PRBC) to immunize mice. Groups of mice were injected intravenously with 5×10^7 fixed PRBCs of *P. yoelii* 17XL, *P. yoelii* 17XNL, or *P. yoelii* 17XL from phenylhydrazine-treated donors (the numbers of parasites in all preparations were similar). Three weeks later, these mice and groups of untreated controls were challenged with lethal *P. yoelii* 17XL (Fig. 1c, d). Prior immunization with erythrocytic *P. yoelii* 17XL did not affect the course of the lethal challenge infections. In contrast, mice immunized with the reticulocytic forms could now resist infection with the lethal variant (Fig. 1c, d).

These results indicated that reticulocytic forms of malaria were more immunogenic than erythrocytic forms; they also implied that immunogenicity was not determined by the parasite *per se* but was rather influenced by the type of RBC infected. Further, as we have shown previously that non-lethal *P. yoelii* 17XNL was a more potent activator of T cells than lethal *P. berghei*² immunogenicity appeared to be related to T cell activating potential. One of the differences between parasitized

* Deceased.

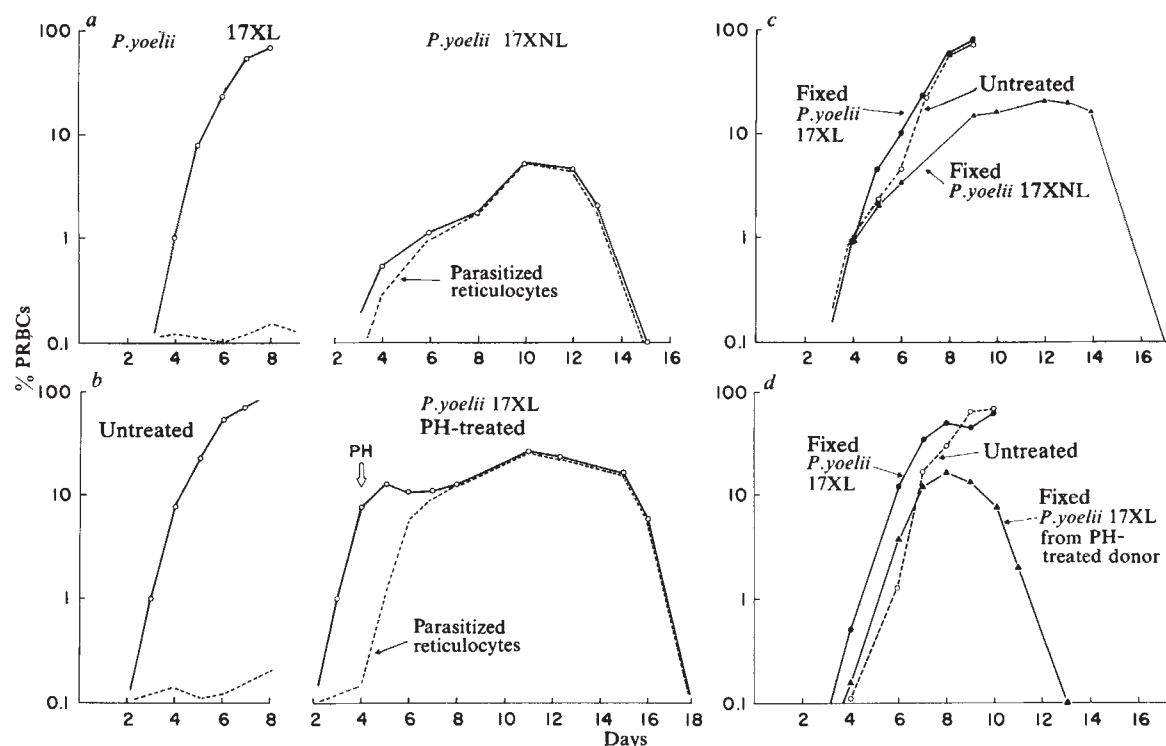


Fig. 1 *a*, Per cent parasitized RBC (erythrocytes and reticulocytes) (○—○) and per cent parasitized reticulocytes (○---○) in 8-week-old male CBA/CaJ mice infected with either 10^5 *P. yoelii* 17XL or 10^5 *P. yoelii* 17XNL PRBC. *b*, Parasitaemias in untreated and phenylhydrazine (PH)-treated CBA/CaJ mice infected with lethal *P. yoelii* 17XL. A single injection of 1.5 mg of phenylhydrazine hydrochloride (Sigma) was administered i.p. 96 h after infection with 10^5 PRBC. *c*, Parasitaemias in untreated CBA/CaJ mice and in mice vaccinated with 5×10^7 formalin-fixed lethal *P. yoelii* 17XL or nonlethal *P. yoelii* 17XNL PRBC and challenged with 10^4 lethal *P. yoelii* 17XL PRBC. *d*, Parasitaemias in untreated CBA/CaJ mice and in mice vaccinated with 5×10^7 formalin-fixed lethal *P. yoelii* 17XL or (reticulocytic) *P. yoelii* 17XL PRBC from PH-treated donors and challenged with 10^4 lethal *P. yoelii* 17XL. Reticulocyte levels were estimated from blood films made on brilliant cresyl blue coated slides counterstained with Giemsa. To prepare the vaccines, CBA/CaJ mice infected and treated with phenylhydrazine (treatment as in *a*) were bled (on day 7 of infection) into cold, heparinized PBS, the blood washed twice at 1,500g for 10 min and resuspended in 0.06% formaldehyde for 10 min at 4 °C. The fixed cells were washed twice, resuspended to the desired cell concentration and injected intravenously (i.v.) into groups of 5 mice. No infections were caused by the fixed cells as judged by blood film examination over a 3-week time after vaccination. The mice were challenged with a live *P. yoelii* 17XL inoculum 3 weeks after vaccination.

Table 1 Red cell preference of malarial parasites and characteristics of infection

Parasite	Red cell preference	Characteristics of infections in CBA/CaJ mice*		
		Duration (days)	Peak parasitaemia (% parasitized RBCs)	Mortality (%)
<i>P. yoelii</i> 17XNL	Reticulocytes	15	5–10	0
<i>P. yoelii</i> 17XL	Mature erythrocytes	7–9	80–90	100
<i>P. vinckei</i>	Mature erythrocytes	7–8	70–80	100
<i>P. berghei</i> (ANKA)	Mature erythrocytes (days 10–14) and reticulocytes†	20–25	50–60	100
<i>P. chabaudi</i>	Mature erythrocytes and some invasion of reticulocytes	15	50–60	30

* 10-week-old CBA/CaJ mice were infected with 10^5 PRBC intraperitoneally (i.p.). Parasitaemias and red cell maturity were estimated from blood films made on brilliant cresyl blue coated slides, counterstained with Giemsa.

† *P. berghei* parasitized reticulocytes appear more mature than most of the reticulocytes infected by *P. yoelii* 17XNL.

erythrocytes and reticulocytes that we felt might be relevant to the degree of immunity induced by these two cell types was the known differences in the level of expression of major histocompatibility complex (MHC) antigens; it is becoming increasingly clear that they play a fundamental part in T cell responses to intracellular parasites¹⁵. Reticulocytes express significant amounts of these antigens^{6,7} but although H-2K and D antigens are present on erythrocytes in the mouse, their density is considerably less than on most other cell types¹⁶.

Initially we compared the expression of H-2K and D and Ia on normal erythrocytes, normal reticulocytes and *P. yoelii* 17XNL infected reticulocytes by indirect immunofluorescence

and fluorescence-activated cell sorting (FACS) analysis. Using monoclonal and monospecific anti H-2 sera, *P. yoelii* 17XNL-infected CBA/CaJ reticulocytes showed markedly enhanced expression on H-2K antigen compared to normal erythrocytes or reticulocytes (Fig. 2a). A similar increase in H-2D antigen was demonstrated on *P. yoelii* 17XNL infected B-10 reticulocytes (Fig. 2b). Expression of H-2K and D antigens was also examined by absorption analysis. *P. yoelii* 17XNL infected CBA/CaJ reticulocytes showed a six- to eightfold increase in the expression of these antigens compared to normal CBA erythrocytes or reticulocytes (data not presented). The increased expression of these antigens could not be attributed

Fig. 2 a-c, FACS analysis of H-2K, H-2D and Ia antigen expression on normal erythrocytes, normal reticulocytes or *P. yoelii* 17XNL infected reticulocytes. **a**, H-2K^k expression on CBA/CaJ (H-2^k) cells detected by use of a monoclonal anti-H-2K^k (clone 11-4.1) from Becton Dickinson. Clone 11-4.1 was derived by hybridization of NS-1 myeloma cells with spleen cells from BALB/c (H-2^d) mice immunized with CKB (H-2^k) spleen cells³⁰. Analysis of cells stained with the FITC conjugate only gave results coincident with background fluorescence shown by unstained samples. **b**, H-2D^b expression on B10 (H-2^b) cells. Antiserum used was ASM34 (B10.A(18R) × A/SnF₁ anti-B10 anti-H-2D^b). **c**, Ia^k expression of CBA/CaJ (H-2^k) cells. **d**, The light scattering (size) distribution of normal and *P. yoelii* 17XNL infected CBA/CaJ reticulocytes.

Methods: To obtain *P. yoelii* 17XNL infected reticulocytes, infected mice (day 7) were bled into cold mouse tonic phosphate buffered saline (MTPBS), washed twice at 1,500g for 10 min and passed through columns of Sephadex G25 and Sulphoethylcellulose (SE23) to deplete white blood cells (WBC)²⁹. This procedure reduced WBC contamination to less than 0.01%. The parasitized RBC were then separated on Percoll-Renograffin density gradients at 20,000g for 25 min in a Sorvall RC5 centrifuge. This enrichment procedure provided a population of cells that was more than 90% parasitized and contained less than 1.5% WBC. The cells were washed twice in MTPBS before being processed for immunofluorescence. Normal reticulocytes were obtained from mice in which a reticulocytosis was induced by repeatedly bleeding from the retro-orbital sinus (0.5 ml at one day intervals). When reticulocyte levels reached 30–50% mice were bled and processed as for parasitized blood. Normal erythrocytes were obtained by bleeding untreated mice and these cells were depleted of WBC before use. For immunofluorescence, aliquots of cells were spun down and resuspended in suitably diluted anti-H-2K, D or anti-Ia sera. After 20 min at 4 °C the cells were washed twice and resuspended in a suitably diluted fluorescein-conjugated goat anti-mouse IgG (Meloy) for 20 min. The cells were washed three times and adjusted to the required cell concentration for examination on a fluorescence activated cell sorter^{31,32} (FACS IV Becton Dickinson). Antiserum used: monoclonal anti-Ia (specificity 2) (clone 11-3.25)³⁰ from Becton Dickinson.

either to an increased cell size, as size judged by light scatter was comparable for normal and parasitized reticulocytes (Fig. 2d), or to an increase in contaminating white blood cells (WBC) since the percentage of WBC was roughly equal (0–1.4%) in control and test populations. We also examined the expression of Ia since erythroblasts have been reported to express Ia-like antigens¹⁷ although erythrocytes lack Ia¹⁸. We failed to detect Ia on normal or *P. yoelii* 17XNL parasitized reticulocytes using monoclonal and monospecific anti-Ia sera (Fig. 2c).

To examine the relationship between the expression of H-2 antigens and the pathogenesis of these infections we compared the expression of H-2K on (1) PRBCs from CBA/CaJ mice infected with different strains of malaria (Fig. 3a), and (2) on PRBCs from strains of mice of varying degrees of susceptibility infected with the same parasite (*P. yoelii* 17XNL) (Fig. 3b). Unlike reticulocytes infected with *P. yoelii* 17XNL erythrocytes infected with the lethal variant showed no significant increase in H-2K expression. *P. chabaudi* infected RBC showed slightly enhanced expression compared to normal RBC while in *P. berghei* infected cells expression was not significantly above background. All cells were collected from CBA/CaJ mice 7 days after infection. We also examined the expression of H-2D on *P. yoelii* 17XL and *P. berghei* PRBCs. No increase was detected (data not shown). Comparison of H-2K on parasitized RBCs derived from different strains of mice infected with *P. yoelii* 17XNL revealed a close correlation between the expression of these antigens and the ability of the host to control the infection (Fig. 3b). In CBA/CaJ mice which are high responders maximal H-2K expression was seen and the parasites were exclusively in reticulocytes (infection lasted 15–16 days). In BALB.K mice, least expression of increased H-2 was seen (Fig. 3b). BALB.K is a low responder—infections lasting 25–26 days. In AKR mice, which is a strain of intermediate susceptibility (duration of infection 20 days), the level of H-2K expression was in between that observed on CBA/CaJ and BALB.K cells.

These results and our previous observations² are compatible with the idea that a parasitized reticulocyte is a more effective inducer of immunity than a parasitized erythrocyte. They further suggest that enhanced expression of H-2K and H-2D antigens may be one of the factors responsible for this immunogenicity. Other factors such as the greater fluidity

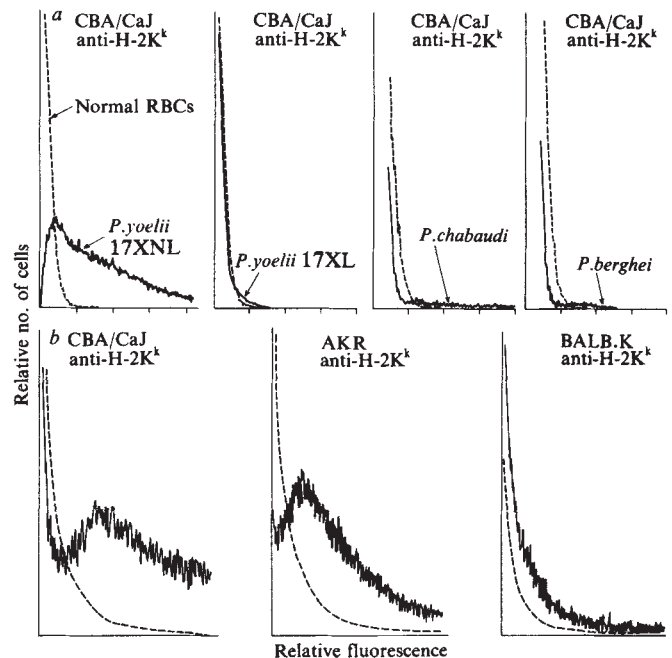
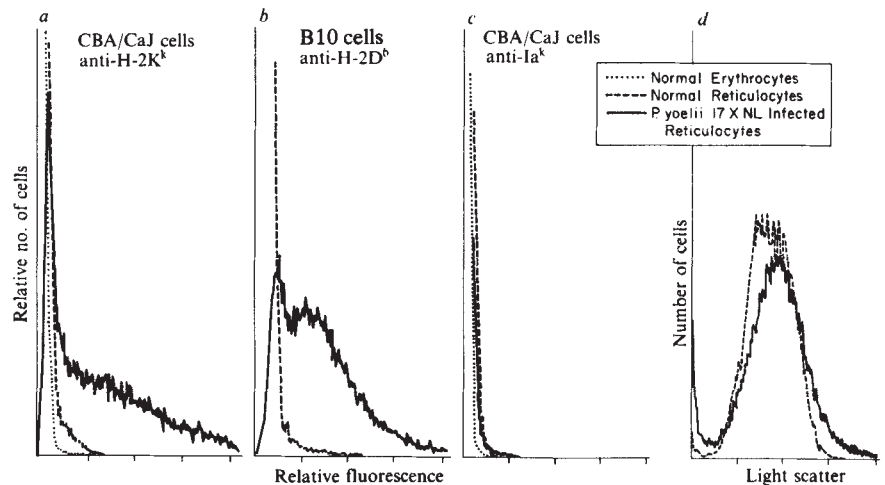


Fig. 3 FACS analysis of H-2K^k expression. **a**, Comparison of H-2K expression on *P. yoelii* 17XNL, *P. yoelii* 17XL, *P. chabaudi* and *P. berghei* (ANKA) infected RBC from CBA/CaJ mice. **b**, Comparison of H-2K^k expression on *P. yoelii* 17XNL parasitized RBC from CBA/CaJ (H-2^k), AKR/J(H-2^k) and BALB.K (H-2^b) mice. All procedures were carried out as described previously (see legend to Fig. 2). Parasitized cells were separated 7 days after infection. The antiserum used was a monoclonal anti-H-2K serum (Y3) provided by Drs E. Lerner and C. A. Janeway. Y3 was derived by hybridization of NS1 myeloma cells with spleen cells from BALB/c (H-2^d) mice immunized with BALB/c (H-2^b) spleen cells. The antiserum cross-reacts with H-2K^k.

of the reticulocyte membrane¹⁹, which may permit more effective expression of parasite antigens, may also facilitate immunogenesis.

The reason why increased H-2K and H-2D expression of the parasitized reticulocyte should enhance immunogenicity

against the malaria antigen is currently being tested. The underlying assumption is that these MHC class I self-antigens constitute a part of the repertoire that some T cells see as foreign. This has clearly been shown to be the case with Ly-2⁺ killer cells^{20,21} and there is evidence that increased expression and turnover of these class I antigens enhances both tumour immunity²² and killer cell activity against TNP-modified self²³. However, Ly-1⁺ helper cells have not been reported to use class I antigens to facilitate their interaction with foreign antigen. The Ly-1⁺ cells are the cells which confer protective immunity against malaria; no significant role for Ly-2⁺ cells has been found²⁴. There is some evidence that suppressor cells may see antigen together with class I antigens^{25,26}. Thus, it may be that the enhanced immunogenicity of parasitized reticulocytes is due to a negative effect on suppressor cells produced by the expression of high levels of class I antigens. This negative effect could be brought about either directly, by inhibiting their triggering or indirectly, by activating cells of the newly described contrasuppressor circuit²⁷.

That reticulocytes may provide a generally more immunogenic presentation of antigens derived from intracellular parasites is not itself a novel concept. Playfair *et al.*²⁸ used phenylhydrazine to induce reticulocytosis and found that when these cells were infected with either *P. yoelii* or *P. berghei* and subsequently labelled with TNP, they induced a better anti-TNP IgM response than did normal reticulocytes. These results indicate that parasitized reticulocytes present exogenous antigens in a more immunogenic fashion to the immune system and are consistent with our finding that they present the parasitic (malarial) antigens in a better fashion as well. This finding brings up the question of whether or not the parasitized reticulocytes bearing the fatal form of the *P. yoelii* also express the same increased amounts of H-2 antigens as do the reticulocytes parasitized with the nonfatal form. Playfair's data suggest that such might be the case since the parasitized reticulocytes presented TNP much better than did normal reticulocytes, indicating that the parasitic infection of the phenylhydrazine-induced reticulocytes made them better carriers of haptens. We are now trying to determine if it is simply the increased H-2 antigens on the reticulocytes that are responsible for their hyperactivity as carriers of antigen. However, even unparasitized reticulocytes carry more H-2 antigens than do mature red blood cells and could thus, in theory, present malaria antigens to the immune system in a more efficient manner.

These results strongly suggest that the type of cell in which malarial parasites grow is an important factor in determining the immune response against them. There is a very good correlation of the expression of high levels of class I MHC antigens with immunogenicity and, if a causal relationship can be found, these results could have far-reaching effects on the methods of preventive immunization, not only against malaria or other parasites, but perhaps also against other kinds of foreign material that would be advantageous for the body to reject.

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A survey of human leukaemias for sequences of a human retrovirus

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Human T-cell leukaemia-lymphoma virus (HTLV) is an exogenous human retrovirus distinct from all known animal retroviruses. HTLV is closely linked to a subtype of adult T-cell malignancies and except for isolated cases, has not been found associated with any other form of leukaemia, lymphoma or other cancers (see refs 1, 2 for review). HTLV can be transmitted to cord blood T lymphocytes *in vitro* and the infected cells exhibit characteristics of transformed neoplastic T cells^{3–5}. We have recently cloned DNA sequences derived from approximately 1 kilobase (kb) of the 5' and 3' termini of the HTLV genome, as well as a 4–5-kb defective HTLV provirus flanked by cellular sequences⁶. The availability of these probes has enabled us to carry out a limited survey of different fresh or cultured cells from patients of different lymphoid and myeloid malignancies for HTLV-related DNA sequences. The results presented here show that cells from all Japanese patients with adult T-cell leukaemia and several patients with various mature T-cell malignancies from elsewhere contained one or more copies of a highly conserved HTLV genome. The infected cells are of clonal origin. Fresh cells from 1 of the 10 myeloid leukaemic patients contained exogenous DNA sequences distantly related to HTLV.

Four different viral probes which represent different regions of the HTLV genome were used: two recombinant plasmids containing 400 and 600 nucleotides, respectively, of R-U5 and R-U3 sequences, a 6-kb fragment of a cloned defective integrated HTLV provirus from the neoplastic T-cell line CR-M2 containing *LTR-evn-pol* and 1 kb of flanking cellular sequences, and a 3-kb fragment containing only sequences of the *env-pol* region. Table 1 summarizes results of DNA from various mature and immature T- and B-cell lines digested with various restriction enzymes and blot-hybridized to different nick-translated probes. One of the enzymes used, *EcoRI*, does not cut within the prototype HTLV genome, thus each *EcoRI* band detected on a Southern blot by HTLV probes represents a single proviral copy at a particular integration site (ref. 7 and our unpublished data). All the mature T-cell lines known to express HTLV antigens and/or infectious virus contained one

or more HTLV genome copies. MO, a T-cell line developed from a patient with hairy cell leukaemia⁸, has been shown to produce a subtype of HTLV distinct from the prototype as demonstrated by the slopes of competitive immunoprecipitation of purified viral p24 (ref. 9). HTLV *env-pol* probes detect in MO DNA sequences which are distinguishable from prototype HTLV by restriction patterns and stringency of hybridization (not shown). Analysis of MO cell RNA in relaxed hybridization conditions revealed hybridizing viral messenger RNA (G.F. *et al.*, unpublished). These results suggest that HTLV from MO differs from the prototype HTLV at the nucleotide sequence level, although it belongs to the HTLV family because its target is also mature T cells and its proteins (p24, p19) are related to those of the prototype HTLV. Another cell line, HUT 78, of a mature T cell that expresses no HTLV antigens or virus particles contained HTLV proviral sequences (R.C.G. *et al.*, unpublished). Several other lymphoid cell lines, including several mature T-cell lines from patients with mycosis fungoides, Sézary syndrome, immature T-cell lines and B-cell lines failed to reveal any HTLV-related sequences (Table 1).

Fresh leukaemic cells from patients with different lymphoid malignancies were screened as described above. DNA from all Japanese patients with adult T-cell leukaemia (ATL) examined (five out of five) contained one or two copies of HTLV provirus, including a patient (T.O.) who had no circulating antibodies against HTLV antigens (Robert-Guroff *et al.*, unpublished). On the other hand, a patient (E.T.) with T-cell acute lymphocytic leukaemia (T-ALL) was found to have antibodies reactive with HTLV antigens¹⁰ even though his leukaemic cells were immature T cells, normally not the target for HTLV infection. By hybridization, his leukaemic cells indeed lacked HTLV DNA sequences. This result suggests that this patient was at one time exposed to HTLV. His disease may have had a different aetiology, or viral sequences may have been deleted from his malignant cells in an event that also resulted in the loss of the mature T-cell markers. Other positive samples include leukaemic cells from an Israeli patient (U.K.) with peripheral diffuse lymphoma from whose cells a virus-producing line has been established^{4,10} (also see Table 1) and a skin biopsy of a Brazilian patient (M.A.) with mycosis fungoides. Several other samples from patients with Sézary syndrome and cutaneous T-cell lymphoma (CTCL), as well as 20 ALL and chronic lymphocytic leukaemia (CLL) samples were negative in this survey.

We conclude that all HTLV-positive fresh leukaemic cells and cultured cell lines are clonal populations of infected cells, because DNA bands greater than genomic size (which therefore must contain viral and flanking cellular sequences) are readily visible (Figs 1, 2). Figure 1 shows a representative DNA blot of samples from two ATL patients hybridized to a HTLV_{R-U5}

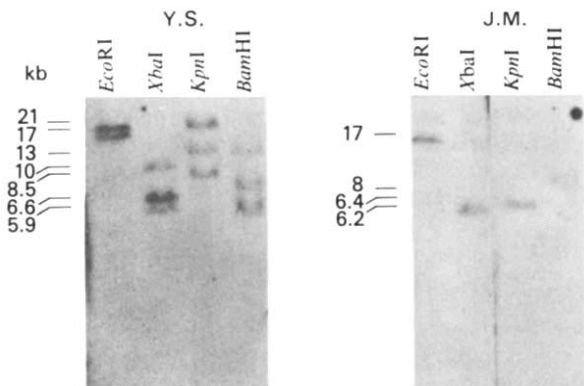


Fig. 1 Integration of HTLV proviruses in fresh leukaemic cells of two Japanese patients with adult T-cell leukaemia. 30 µg of cellular DNA from leukaemic cells of two ATL patients were digested with *EcoRI*, *XbaI*, *KpnI* and *BamHI* and analysed by electrophoresis in a 0.8% agarose gel. The gel was blotted onto nitrocellulose filter paper and the filter was hybridized to nick-translated ³²P-labelled HTLV_{R-U5} sequences.

probe. DNA from Y.S. and J.M. contained two and one copies of HTLV, respectively, as shown by the number of *EcoRI* viral fragments. It is of interest that the enzymes *XbaI*, *KpnI* and *BamHI*, which cut within the prototype HTLV genome, generated a single DNA fragment in the J.M. sample. As the R-U5 probe detects sequences within the long terminal repeat (LTR), which are present twice in a complete provirus, our data suggest that J.M. contained a single defective provirus lacking at least one LTR. Defective proviruses or combinations of nondefective and defective proviruses have been found in avian leukosis virus-induced lymphomas^{11,12} and bovine leukaemia virus-associated lymphomas¹³. This result also indicates that not all leukaemic cells from HTLV-positive leukaemias will yield fully infectious viruses.

Apart from HTLV_{MO}, which is greatly divergent from the prototype HTLV⁹, all other HTLV isolates so far obtained seem to be closely related to each other in their proteins and nucleotide sequence homology. We had proposed to classify HTLV_{MO} as HTLV-II and all other HTLV strains as HTLV-I subgroups⁹. For a more sensitive comparison, we examined DNA from all the HTLV-positive fresh cell samples and cell lines derived from primary tumour cells by Southern hybridization, using the enzymes *BamHI* and *PstI*, which excise internal fragments from within HTLV_{CR} (ref. 6). As shown in Fig. 2, the HTLV genomes as present in patients from Japan, USA, Israel, Brazil and the Caribbean all contain an internal 1.05-kb *BamHI* fragment and a 2.6-kb *PstI* fragment as detected by the HTLV *env-pol* probe. This result indicates that the different HTLV strains of subgroup I are highly conserved throughout many geographical regions.

Although seroepidemiological studies revealed an association of HTLV strictly with T-cell malignancies and the prototype HTLV infects preferentially mature T cells *in vitro*, we were interested to see if cloned HTLV probes might detect cross-reactive sequences in cells of other haematopoietic neoplasias.

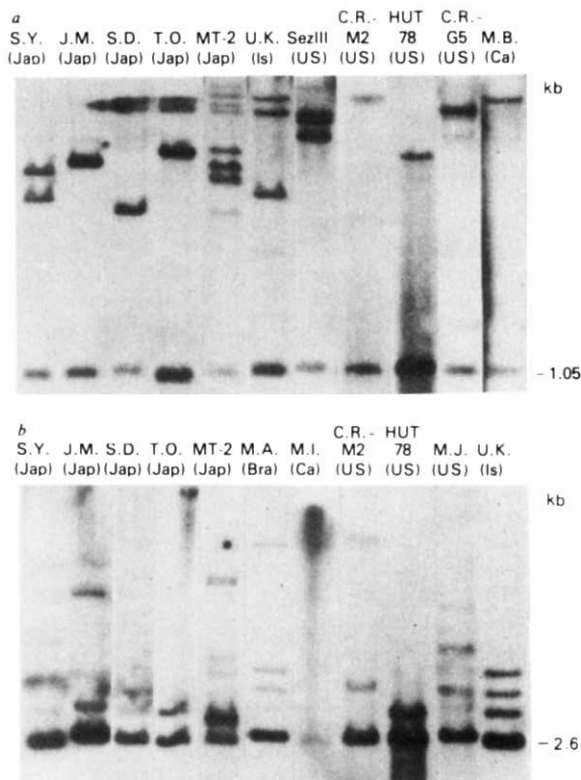


Fig. 2 Conservation of the HTLV genomes present in diverse geographical areas. DNA from fresh leukaemic cells or cell lines from HTLV-positive patients from different areas of the world was digested with the restriction enzymes *BamHI* (a) and *PstI* (b) which cut more than once within HTLV_{CR}, and blot hybridized to a HTLV *env-pol* probe. The places of origin are: Jap, Japan; Is, Israel; US, United States; Ca, Caribbean; Bra, Brazil.

We examined fresh leukaemic cells from 10 patients with acute (AML) and chronic (CML) myelogenous leukaemia. All except one sample were negative. DNA from fresh cells of one CML patient in blast crisis revealed a 4.7-kb *Xba*I band and a 10-kb *Kpn*I band hybridized to HTLV LTR probes after a 2-week exposure of the autoradiogram (Fig. 3). A duplicate filter hybridized to pHT-3, a recombinant plasmid containing a single copy cellular gene¹⁴, yielded a different array of bands characteristic of pHT-3, indicating the bands detected by HTLV-LTR were not due to pBR322 cross-hybridizing sequences (Fig. 3). However, HTLV probes that lack LTR sequences did not hybridize to this DNA (not shown). This result suggests several possibilities: (1) the leukaemic cells from this patient contained

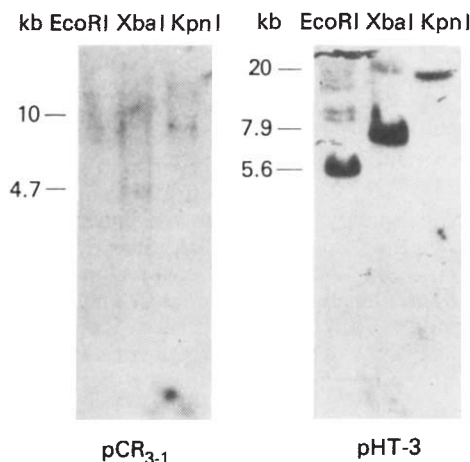


Fig. 3 Detection of HTLV-related DNA sequences in leukaemic cells of a CML patient. Duplicate DNA filters were hybridized with labelled pCR3-1, a recombinant plasmid containing HTLV_{R-U3} sequences (a) and pHT-3, a recombinant plasmid containing a single copy cellular gene¹⁴ (b).

Table 1 Survey of human lymphoid cell lines for HTLV provirus

Cell line	Diagnosis	Cell type	HTLV markers	Proviral copy no.
HTLV positive				
CR-M2	CTCL (MF)	Mature T cells	+	2-3
CR-G5	CTCL (MF)	Mature T cells	+	1
HUT 78	CTCL	Mature T cells	-	1
SEZ III	CTCL	Mature T cells	+	3
MB	CTCL	Mature T cells	+	1
MI	T-LCL	Mature T cells	+	>3
UK	PTCL	Mature T cells	+	2
MJ	CTCL (MF)	Mature T cells	+	>3
WA	DML	Mature T cells	+	1
MT-2	ATL	Mature T cells	+	>3
TK	PL	Mature T cells	+	>3
BK	Healthy	Mature T cells	+	1
HK	Healthy	Mature T cells	+	>3
MO	THCL	Mature T cells	+	>1
HTLV negative				
HPB-MLT	T-LML	Immature T cells	-	-
HPB-ALL	T-ALL	Immature T cells	-	-
CR-B	CTCL (MF)	B cells	-	-
8392	T-ALL	B cells	-	-
8402	T-ALL	Immature T cells	-	-
CCR-SB	T-ALL	B cells	-	-
HSB2	T-ALL	Immature T cells	-	-

CTCL, cutaneous T-cell lymphoma; MF, mycosis fungoides; T-LCL, T-lymphosarcoma cell leukaemia; PTCL, peripheral T-cell lymphoma; DML, diffuse mixed lymphoma; ATL, adult T-cell leukaemia; PL, persistent lymphocytosis; T-LML, leukaemic phase of T-cell lymphoma; T-ALL, T-cell acute lymphocytic leukaemia; THCL, T-cell variant of hairy cell leukaemia. T.K., B.K. and H.K. are family members (mother, father and brother, respectively) of a Japanese ATL patient, S.K.¹⁶. MO is releasing a virus that is distantly related to the prototype HTLV (see text). As this virus has related nucleic acid sequences, but a different restriction enzyme map, the restriction mapping criteria for integrated proviral copies cannot be applied.

a small population of T cells which harboured HTLV; (2) HTLV is not entirely T-cell trophic and is involved in some myeloid malignancies; (3) the HTLV probe is detecting cross-reactive sequences of a distinct retrovirus associated with some myeloid leukaemias. The weak signal intensity obtained only with HTLV-LTR sequences and the apparent monoclonality of the infected cells tend to support the last possibility.

Thus, we have used cloned HTLV nucleic acid sequences to survey a number of human haematopoietic cell lines and fresh leukaemic cells from patients with different lymphoid and myeloid malignancies for DNA sequences related to HTLV. We draw several conclusions from these studies:

(1) Cells from some patients of mature T-cell malignancies, including all patients with ATL, contained one or a few copies of HTLV provirus. In some cases, a single defective provirus was present, suggesting that not all HTLV-positive leukaemic cells would yield fully infectious virus.

(2) The surveys by molecular hybridization and by serology do not correlate in every case. We believe this discrepancy to be due to the following situations: patients whose leukaemic cells have proviral sequences but do not express viral antigens (for example, due to defective viral genomes) and thus do not produce antiviral antibodies; alternatively, patients who have incidental HTLV infection of their T lymphocytes or some other target cell and are therefore positive for antibodies, but whose leukaemic cells do not contain integrated HTLV proviral sequences and therefore whose disease is probably not linked to HTLV.

(3) The HTLV strains can be divided into two subgroups. HTLV-I is a virus whose sequences are highly conserved despite extensive distribution throughout different regions of the world. To date, HTLV-II has only one isolate, the MO strain, which differs from HTLV-I in sequence homology and restriction enzyme mapping.

(4) The tumour cells are clonal expansions of single infected cells. Monoclonality is a common feature of tumours induced by the chronic leukaemia viruses, but not by the rapidly transforming *onc* gene-containing viruses. It has been proposed that activation of cellular genes is one mechanism of leukaemogenesis by the chronic leukaemia viruses¹⁵. Activation of a cellular gene (HT-3) specifically involved in mature T-cell proliferation was observed in all HTLV-infected cells¹⁴. It would be of great interest to determine whether there is a common integration locus for HTLV and whether activation of cellular genes, including HT-3, occurs in the vicinity of the provirus integration sites.

(5) We detected weakly cross-reactive sequences, which may represent a distantly related virus, in cells from one CML patient. This result suggests that although the prototype HTLV is specifically associated with mature T-cell malignancies, cloned HTLV sequences may be useful in detection of other cross-reactive putative human retroviruses involved in other malignancies.

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Entrapment of animal cells for production of monoclonal antibodies and other biomolecules

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Animal cell technology is attracting considerable interest because of the capacity of animal cell cultures to synthesize or transform complex compounds such as virus vaccines, immunochemicals, hormones or enzymes¹. For the growth of surface-dependent cells, microcarrier technology is gaining importance². Here, we have attempted to immobilize surface-independent cells, normally grown in suspension, by entrapping them in polymer microbeads. Such entrapment should give increased stability to the normally fragile animal cells, allow for high cell densities to be achieved within the beads and make such preparations suitable for continuous operation. At the same time, the need for separation of the desired product from the cells is obviated. With the model systems studied, we showed that hybridoma, as well as other cell lines entrapped in agarose microbeads, remained viable. Both immunoglobulins and lymphokines were exported through the microbeads into the medium for 1–3 weeks, at levels corresponding well to those produced with free cells.

Previous studies had demonstrated that animal cells can be entrapped in a viable state in alginate beads, providing preparations in which the cells are either embedded throughout the network of the bead³ or kept within the microcapsules, which are characterized by a shell of alginate–polyethyleneimine⁴. A drawback of this technique, when applied to animal cells, is the requirement for positive counter ions to keep the polymeric network intact, as this may adversely affect viability and growth of animal cells and/or the fragile nature of such preparations. Furthermore, larger biomolecules, for example, monoclonal antibodies, cannot diffuse out through the polymeric network,

thus preventing their use for continuous processes. In our alternative technique for circumventing these problems, the animal cells are entrapped in agarose beads, the latter formed on cooling a cell–agarose suspension in oil medium⁵. By using oil such as paraffin as a suspension medium, the cells are not harmed, in contrast to normally applied organic solvent media⁶. When applying this technique, agarose microbeads, usually made up of 2.5% agarose, were prepared with an average size of 80–100 μm . As seen from Fig. 1, entrapped mouse–mouse hybridoma cells are evenly distributed within the agarose network. After 1 week of incubation, clusters of cells appeared in the beads due to cell growth.

Table 1 Production of IgG2A κ against herpes simplex type 2 glycoprotein by immobilized hybridoma LSP21 cells

Day	Reciprocal dilution	
	Expt 1	Expt 2
1	256	362
2	512	1,024
3	256	1,024
4	512	ND
5	512	ND
7	256	ND

Anti-herpes simplex virus (HSV) production was measured with an enzyme-linked immunosorbent assay method. First the formed HSV antigens were allowed to bind to specific rabbit anti-HSV immunoglobulin (Dako) immobilized to Nunc immunoplates. In the antigen-containing wells, media or medium dilutions were incubated for 30 min at 37 °C. After washing, peroxidase conjugated anti-mouse IgG (Miles–Yeda 61–204) of 1/600 dilution was added. After further washing, the presence of peroxidase was determined by adding a staining solution containing 40 mg 1,2-phenylenediamine dihydrochloride and 20 μl of 30% H_2O_2 per 100 ml. The reaction was stopped by adding 2 M sulphuric acid and the colour which developed was measured at 492 nm. In expt. 1, LSP21 cells were entrapped yielding 6.2×10^6 cells per ml microbeads containing 2.5% agarose. 20 ml of the microbeads were cultivated in a 250-ml spinner vessel and agitated at 100 r.p.m. in 100 ml medium. About half of the medium (RPMI 1640, supplemented with 10% heat-inactivated FCS) was replaced daily by sedimentation and decantation. In expt 2, LSP21 cells were entrapped yielding 4.3×10^6 cells per ml of microbeads containing 1.2% agarose. Cultivation took place in plastic roller bottles (N. 3027, Falcon) each containing 50 ml of beads suspended in 250 ml of growth medium. The rolling speed was 5 r.p.m. 200 ml of the medium was replaced daily. The average titre of bottle cultures was 1:128. ND, not detected.

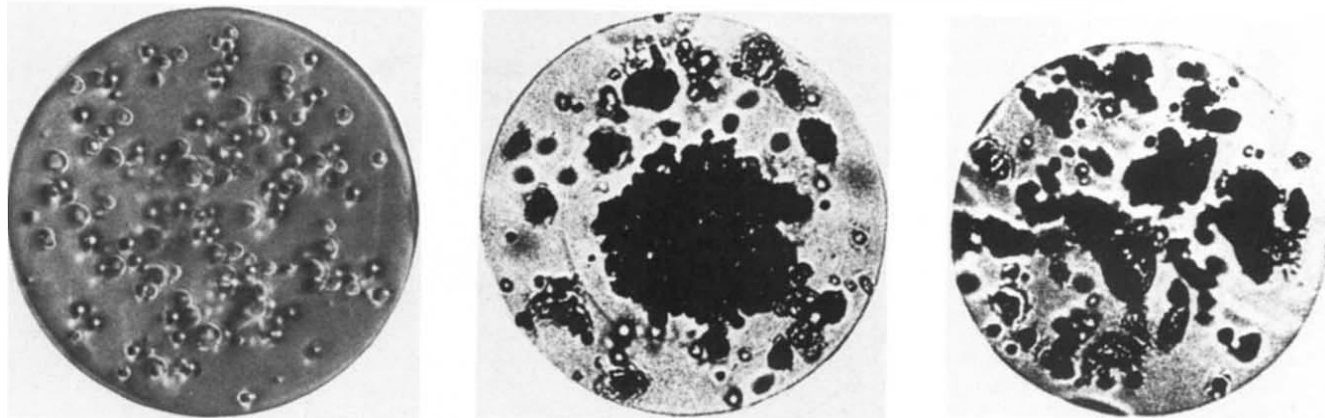


Fig. 1 Entrapped hybridoma cells, LSP21, 6×10^6 cells per ml of beads. The individual cells at time zero (left) can easily be recognized despite the somewhat darker background than in the two pictures showing cell clusters developed after 6 days of culture. The bead size is ~ 80 – $200 \mu\text{m}$. Agarose (Sigma no. A 4018) in phosphate-buffered saline (PBS) without Ca^{2+} and Mg^{2+} at a concentration of 5%, was autoclaved and stored at room temperature. Before use the gel is melted at 70 °C, then cooled to 40 °C and mixed with the cells suspended in an appropriate volume of growth medium. The mixture is poured into a 250-ml round-bottom glass centrifuge tube containing an equal volume of paraffin oil (no. 7174, Merck, Darmstadt) previously washed with PBS and autoclaved. The liquids are emulsified at room temperature with a Vibromixer E1 fitted with a vibrator plate P1, 54 mm diameter (Chemap), to the desired bead size. The mixing vessel is then cooled in an ice bath for 5 min and 50 ml of growth medium added. After another 10 min at room temperature, the tube is centrifuged at 1,200g for 10 min. The oil phase is removed by suction and ~ 100 ml of growth medium is added. After mixing with a magnetic stirrer or with the vibrator plate, the suspension is recentrifuged and the remaining oil removed. The microbeads are transferred to the cultivation vessel and further processed.

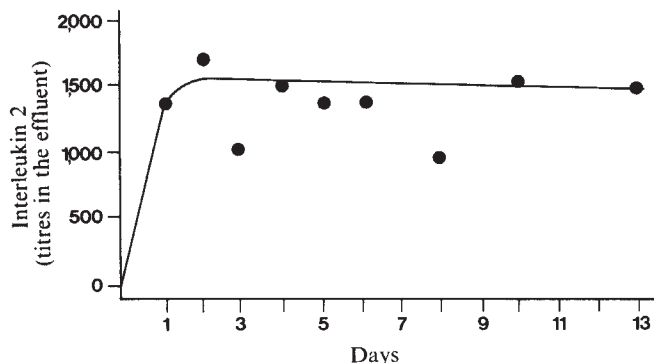


Fig. 2 Continuous production of interleukin 2 by entrapped lymphoblastoid MLA 144 cells, which were entrapped yielding 2×10^7 cells per ml 2.5% agarose beads. 6 ml of the beads were cultivated in a 500-ml spinner vessel and agitated at 100 r.p.m. in 200 ml growth medium, RPMI 1640, supplemented with 5% heat-inactivated fetal calf serum (FCS). About 80% of the medium was replaced daily by sedimentation and decantation. The average titre of the bottle cultures was about 1,500. The interleukin 2 assay is based on an indicator cell line which is absolutely dependent on the presence of interleukin 2⁸. The rate of growth is dependent on the concentration of interleukin 2, without which cells die within the test period. For performing the test, 100 μ l of sample dilutions were dropped into the wells of 96-well microplates (no. 167008, Nunc) and 100 μ l of RPMI 1640 supplement with 10% FCS containing 10,000 indicator cells was added. The plates were then incubated for 2 days at 37°C in a humidified atmosphere containing 5% CO₂. After incubation, cells were fixed at 4°C by the addition of glutaraldehyde (0.2% final concentration) for 30 min after which the supernatant was removed. The cells were stained by the addition of 100 μ l of a freshly prepared and filtered solution of 2% Giemsa in distilled water. The staining period was 3 h at 37°C. After this treatment, the cells were rinsed with PBS to remove excess dye. For measurement, the dye is eluted with 100 μ l of a mixture of equal volumes of ethanol and 0.1 M NaH₂PO₄. After agitation, the plates are measured directly in a Titertek multiscan photometer at 620 nm. Activity is evaluated using the integral of the dose-response curve.

The same entrapped hybridoma cells were also tested for their capacity to form monoclonal IgG2A κ antibodies against herpes simplex type 2 glycoprotein. As seen in Table 1, daily removal and assay of the supernatant for a period of 1 week showed a more or less unchanged production capacity, comparable with that of free cells grown as suspension cultures in bottles. Note that the two kinds of experiments refer to cultivation of cells in spinner flasks (using 2.5% agarose beads) and in roller flasks (using 1.2% agarose beads) and are thus not quite comparable. Several other hybridoma cell lines tested, such as human-mouse hybridoma forming IgG1 κ against anti-human influenza A nucleoprotein, could also be conveniently entrapped with no impairment of their monoclonal antibody formation capacity.

A cell line producing interleukin 2, known to stimulate lymphocyte growth, was tested as a model system for the production of other biomolecules of medicinal interest. The gibbon lymphoblastoid cell line in question had a high spontaneous release of interleukin 2⁷. As Fig. 2 shows, continuous production of interleukin 2 occurred over the 13-day period tested, when the immobilized cells were kept in spinner flasks. In this experiment a high cell concentration (2×10^7 cells per ml beads) was chosen. With conventionally grown lymphoblastoid cells in spinner flasks, such high cell densities cannot be obtained, which shows that the use of agarose entrapment techniques allows desired cell densities to be obtained artificially. This may be a great advantage because it allows the construction of efficient systems which can produce higher concentrations of the desired biomolecules in question than found with conventional suspension cultures or bottle flasks. In addition, as demonstrated for hybridoma cells lines (Fig. 1), the entrapped cells may subsequently be brought to grow further within the microbeads.

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***Caulobacter* flagellin mRNA segregates asymmetrically at cell division**

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Molecular processes which promote the spatial localization of subcellular components are fundamental to cell development and differentiation. At various stages in development unequal segregation of molecular information must occur to result in the differentiated characteristics which distinguish cell progeny. Biological attributes of the dimorphic bacterium, *Caulobacter crescentus*, provide an experimental system permitting examination of the generation of asymmetry at the molecular level¹. When a *Caulobacter* cell divides, two different daughter cells are produced—a motile swarmer cell with a polar flagellum and a non-motile cell with a static appendage referred to as a stalk. The two cell types are distinct with respect to surface morphology²⁻⁵, developmental potential^{1,6}, protein composition⁷ and biosynthetic capabilities^{8,9}. One of the more conspicuous manifestations of asymmetric expression of macromolecules in this system, the flagellum, has been studied extensively^{3,10-13}. We have cloned the flagellin genes of *Caulobacter*¹⁴ and report here the use of these sequences as probes to demonstrate that (1) the level of flagellin mRNA is regulated during the cell cycle in a pattern coincident with flagellum polypeptide synthesis and (2) flagellin mRNA synthesized before cell division is segregated with progeny swarmer cells. This provides molecular evidence of specific partitioning of an mRNA at the time of cell division.

The flagellum of *Caulobacter* is assembled during development of the swarmer cell³. The filament is composed primarily of two immunologically related flagellin monomers, one of 25,000 molecular weight (M_r), the other of 27,500. Synthesis of flagellin 27,500 and 25,000 M_r polypeptides and their assembly begin before cell division and the flagellum segregates with the progeny swarmer cell (see schema, Fig. 1). 25,000- M_r flagellin polypeptide synthesis persists in the swarmer cell after cell division whereas 27,500- M_r synthesis is shut off. The level of 25,000- M_r flagellin synthesis in the swarmer cell decreases with a half life of 4 min and is unaffected by the drug rifampicin^{6,10}. The expression of flagellin genes as well as that of other polypeptides synthesized at different times during the cell cycle⁷ is believed to be controlled at the level of RNA transcription¹⁵. Like all bacterial mRNAs, the functional half life of the 27,500- M_r and 25,000- M_r mRNAs is short, 2.5 and 4 min, respectively. As rifampicin inhibits the initiation of RNA transcription in *Caulobacter* and no new flagellin mRNA is made in the swarmer cell, these findings have been interpreted to indicate functional segregation of flagellin mRNA at the time of cell division¹⁵⁻¹⁷.

Using cloned flagellin gene fragments, we have examined the pattern of flagellin mRNA synthesis and segregation during the cell cycle. Several subclones were constructed from clones pCA160 and pCA110, which were first prepared using a flagellin gene probe derived from the immunoprecipitation of polyribosomes with anti-flagellin antibody¹⁴. Two subclones, pCA161 and pCA111, were chosen for experiments described

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here. pCA161 contains a 0.9-kilobase (kb) insert which encodes three-quarters of the structural gene sequence for 25,000-*M_r* flagellin polypeptide. pCA111 is a 2.2-kb fragment which contains the entire gene for the 27,500-*M_r* flagellin polypeptide (P. R. Gill and N. A., manuscript in preparation).

Caulobacter cells were synchronized¹⁸ and aliquots removed at 10-min intervals throughout the 120-min cell cycle. Total RNA was purified from each sample and immobilized on nitrocellulose according to the 'dot-hybridization' procedure¹⁹. Plasmid DNA from pCA111 and pCA161 or purified DNA inserts were labelled by nick-translation²⁰ and hybridized in probe excess to the isolated RNA. The amount of labelled probe hybridizing to each RNA preparation fixed to nitrocellulose filters was then measured by scintillation spectrometry. Figure 1 shows the aggregate results of several such experiments. The hybridization of flagellin probes with mRNA isolated during the cell cycle demonstrates that the relative abundance of flagellin mRNA varies during *Caulobacter* development. The temporal pattern of RNA synthesis coincides with that of expression of flagellin polypeptides demonstrated previously³, with the major period of flagellin polypeptide and

mRNA synthesis occurring before cell division, when the cell is an elongated predivisional intermediate.

Because *Caulobacter* cells do not form a septum before division²¹, the predivisional cell is essentially a single cellular unit expressing the differentiated characteristics of both of the incipient daughter cells. If no new mRNA synthesis is initiated in the swarmer cell, as indicated by the referenced experiments described above, flagellin mRNA must be functionally segregated with the swarmer cell at cell division. This functional segregation might occur by compartmentation of the flagellin mRNA in the swarmer cell. Alternatively, flagellin mRNA may be present in both progeny cell types, but flagellin polypeptide synthesis in stalked cells might be regulated at the post-transcriptional or translational level.

These alternatives were tested by analysing the amount of flagellin mRNA present in daughter cells by hybridization with cloned flagellin sequences. *Caulobacter* cells were synchronized and several samples collected at stalked and predivisional stages. After division, the daughter swarmer and stalked cells were re-isolated by density gradient centrifugation⁶, and all the samples were analysed for the presence of flagellin mRNA using fragments from pCA111 and pCA161, as described in Fig. 1 legend.

Figure 2 depicts the results of several experiments which measured the relative amount of flagellin mRNA in the predivisional cells and in each of the separate progeny cell populations. The analysis is performed using a constant amount of RNA and the level of hybridization detected in the predivisional cell was set at one. The new stalked and swarmer cells in effect have approximately one-half the complement of total RNA present in the predivisional cell. If all of the flagellin mRNA synthesized in the predivisional cell is completely segregated, the level of hybridization to message in the daughter swarmer cell should approach 2, that is twice that in the mother predivisional cell. Levels greater than 1 suggest that a rapid degradation of flagellin-specific mRNA in stalked cells does not occur. As Fig. 2 indicates, we observed a range of flagellin mRNA levels in the swarmer cell which average greater than 1; in some individual experiments the level of hybridization to mRNA approached 2. We have found that the synchronization procedure produces swarmer cells which vary in age by as much as 15 min⁷. Thus, the variation in RNA levels in progeny swarmer cells immediately after synchronization is expected. This,

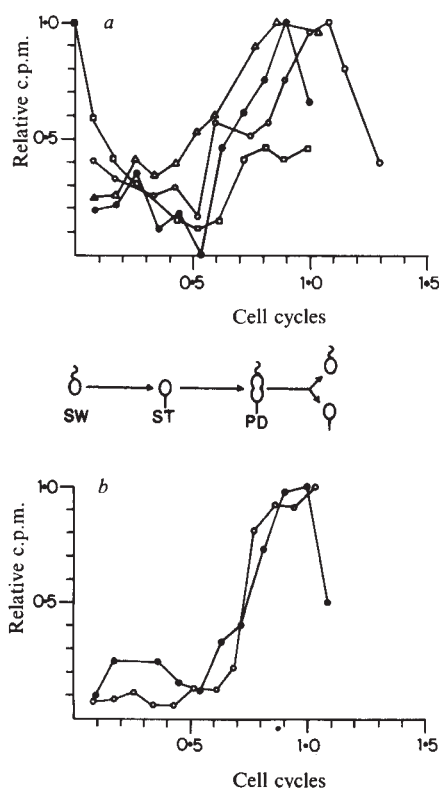


Fig. 1 'Dot-hybridization' of cloned flagellin genes to total RNA isolated from synchronous cell populations. *a*, pCA161; *b*, pCA111 (input radioactivity, 1.5×10^6 c.p.m.; specific activity, 10^7 – 10^8 c.p.m. per μ g). *Caulobacter* cells, CB15, were synchronized¹⁸ and aliquots collected during the cell cycle. RNA was isolated from each aliquot²⁷ and 6 μ g from each sample was fixed directly onto nitrocellulose¹⁹. Each symbol represents a series of RNA isolated from separate cell synchronies and hybridized with nick-translated²⁰ clones. Following hybridization each dot was cut out and the amount of radioactivity bound was determined in a liquid scintillation spectrometer (0.5 – 1.5×10^3 c.p.m.; background 30 c.p.m.). The time of the cell cycle was normalized to 1.0 with respect to cell division, which occurred at 120 min. The maximum radioactivity in each experiment is expressed as relative c.p.m. Each symbol represents hybridization with RNA preparations isolated from a different synchronized cell population. The RNA concentration used was within the linear range of response to labelled probe. Control total RNA preparations from chick oviduct cells and *Trypanosoma brucei* did not show hybridization to either probe. All synchronization was monitored by light microscopy^{15,16}. The diagram between *a* and *b* is the *Caulobacter* cell cycle: SW, motile swarmer cell; ST, non-motile stalked cell; PD, elongated predivisional cell.

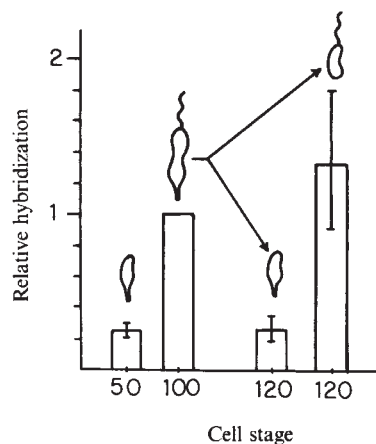


Fig. 2 'Dot-hybridization' of cloned flagellin genes to RNA isolated from progeny cell types. *Caulobacter* cell cultures were synchronized and two aliquots were collected at stalked cell (50 min) and predivisional cell (100 min) stages. At division the synchronous population was synchronized⁶ and progeny swarmer cells and stalked cells collected (120 min). RNA isolated from the cells representing these four cell types, was hybridized and quantitated as described in Fig. 1 legend. The radioactivity bound to the RNA is normalized to 1.0 relative to the amount detected in the predivisional cell. Radioactive input, detected levels and background were similar to Fig. 1. The bars represent the average from three experiments using both pCA161 and pCA111; the range of values from these experiments is indicated.

however, does not compromise the observation that significant levels of message are present only in the swarmer cell (Fig. 2). These results indicate that the asymmetric expression of flagellins is mediated through compartmentation of flagellin mRNA in the swarmer cell. How this occurs is not known, although hypotheses for its mechanism of action can be tested. For example, the physical properties of *Caulobacter* nucleoids suggest that flagellin mRNA could be localized as a result of differential transcription of flagellin mRNA from only one of the chromosomes found in the predivisional cell. DNA replication in *Caulobacter* occurs only during the stalked stage⁸ and Evinger and Agabian^{16,18,22} have shown that stalked and swarmer cell chromosomes can be differentiated on the basis of sedimentation properties and chromosome-associated proteins. Completely differentiated stalked and swarmer nucleoids can be isolated from the predivisional cell²². We have previously suggested that changes in chromosome structure during the *Caulobacter* cell cycle can effect selective transcription^{18,22}. If transcriptional activation of flagellin mRNA is indeed associated with these changes in nucleoid structure, segregation of the nucleoid itself with its altered template specificity could cause segregation of flagellin mRNA.

Alternatively, if both stalked and swarmer cell nucleoids are effective templates for flagellin mRNA synthesis, asymmetric segregation would require that flagellin mRNA be compartmentalized somehow within the predivisional cell. DNA-membrane interactions or ribosome-mediated binding of flagellin mRNA to the cell membrane could provide such a mechanism. Consistent with this, flagellin mRNA has a relatively long functional half life^{6,10}, possibly as a result of protection by complexing with membrane-bound polyribosomes.

Compartmentation is more complex than simply sequestering mRNA in the swarmer cell. In addition to being a swarmer cell-specific protein, flagellin is assembled in an explicit polar cell location. In mechanisms of flagellin mRNA segregation, one may then additionally account for the site-specific assembly of the polypeptide. We have suggested and provided some evidence for the presence of a specialized region of the cell membrane which coordinates the expression of polar structures⁵. Interactions between this region and DNA, perhaps at a specific site on the chromosome, or with mRNA via ribosome binding, could also account for site-specific expression of the flagellum.

Previous studies with *Caulobacter* demonstrated the requirement of *de novo* RNA synthesis during development^{2,15} and a variety of stage-specific polypeptides have been described⁷ whose expression supports the hypothesis of control by transcriptional activation of gene sequences at discrete intervals of the cell cycle. However, no direct evidence of specific transcriptional control has been demonstrated for any *Caulobacter* cell cycle genes. Figure 1, in concert with the direct requirement of *de novo* RNA synthesis⁶, supports the contention that flagellin gene expression is mediated at the transcriptional level, although a stage-specific rapid degradation of mRNA cannot be formally ruled out. Information concerning how changes in template specificity are controlled in prokaryotic systems is limited. RNA polymerase isolated from several stages of the cell cycle demonstrated no significant alterations in polypeptide composition¹⁷. Thus, subunit modification of polymerase which leads to regulation of transcription during phage infection²³ and sporulation²⁴ is an unlikely mechanism for control of flagellin mRNA transcription. The effects of superhelical density of DNA on the control of transcription in other systems has been suggested^{25,26}. Template specificity of developmentally regulated genes in *Caulobacter* may be related to the structural and biochemical changes in the nucleoid during development^{18,21,27}. These and other factors which can influence the temporal and spatial control of flagellin gene expression can be directly tested as a result of the cloning of flagellin genes.

The formation of asymmetry is a fundamental question in the exposition of developmental processes. One of the more elusive aspects of this problem concerns the nature of the

determinants which specify intracellular location. The data presented here identify mRNA as one of the molecules involved in the regulation of asymmetric expression of flagellins in *Caulobacter*.

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Facile transition of poly[d(TG)·d(CA)] into a left-handed helix in physiological conditions

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The DNA polymer d(GC)_n·d(GC)_n can undergo a transition from the usual right-handed 10.4 base pairs (bp) per turn B form to a novel left-handed 12 bp per turn Z form in response to altered environmental conditions¹⁻⁴. Several other alternating purine-pyrimidine DNA polymers with modified bases have been shown to undergo transitions from B to Z conformations, with varying degrees of difficulty⁵⁻⁹. We report here that the unmodified DNA polymer d(TG)_n·d(CA)_n readily undergoes a transition to a Z conformation when subjected to unwinding torsional stress in ionic conditions that are close to physiological. By using a two-dimensional gel electrophoresis system, we have determined both the critical free energy of supercoiling that is required to initiate the transition and the free energy of supercoiling that is required to maintain this polymer in the Z form.

Duplex DNA oligomers of the sequence d(TG)_n·d(CA)_n were cloned by blunt-end ligation into the *Sma*I site of a specially constructed 2.2-kilobase (kb) cloning vector (pDPL6), which contains the origin of replication and ampicillin resistance gene of pBR322 and a compact 36-bp polylinker region containing unique restriction sites for *Cl*aI, *H*indIII, *X*baI, *S*maI, *B*amHI and *S*alI. Redigestion of chimaeric plasmids with *S*maI before transformation of *Escherichia coli* HB101 resulted in a high (>70%) yield of clones containing plasmids with insertions. Individual insertions of up to 120 bp were sequenced by chemical cleavage^{10,11} using restriction sites within the polylinker for end-labelling.

Partially relaxed mixtures of topoisomers from each clone were generated by assembling limited numbers of nucleosome cores onto purified plasmid DNA and then treating the resulting

minichromosomes with chicken topoisomerase I. The relaxed minichromosomes were deproteinized by treatment with pronase and SDS¹². Electrophoresis of the partially relaxed plasmids revealed an accumulation of topoisomers in a specific region of the distributions produced by plasmids containing segments of $d(TG)_n \cdot d(CA)_n$ (Fig. 1, lanes 1*b*, *c*, 2*b*, *c*). A similar accumulation was not observed in the sets of topoisomers generated by unmodified vector, or by similarly constructed plasmids containing different lengths of the isostructural polymer $d(TC)_n \cdot d(GA)_n$ (Fig. 1, lanes 3*a-c*, 4*a-c*). This result indicates that a sequence-dependent structural transition occurs within $d(TG)_n \cdot d(CA)_n$ segments when the unwinding torsional stress in the DNA helix exceeds a critical value.

Figure 2 illustrates an analysis of the topoisomer series of pDPL6 containing 60 bp of $d(TG)_n \cdot d(CA)_n$ (pDHf14, Fig. 1, lane 2*c*) in greater detail, by electrophoresis in a second dimension in the presence of chloroquine¹³. In the presence of this ligand the structural transition within the inserted segments of the topoisomers that accumulated as single bands in the first dimension is reversed, permitting them to be resolved.

To quantitate the transition we define the topoisomer number of a species to be $(\alpha - \alpha_0)$ where α is the topological winding number of the species and α_0 is the topological winding number of the topoisomer having the lowest mobility in the first dimension of electrophoresis. Note that the species having α_0 topological turns migrates more slowly than the nicked circular form of the plasmid (Fig. 2). These plasmids have contour lengths within an order of magnitude of the random coil segment length of DNA in the conditions of electrophoresis¹⁴. It is therefore probable that nicks create preferred sites of kinking which could cause the observed increase in electrophoretic mobility of the nicked form relative to the most fully relaxed closed circular form.

From results similar to those shown in Fig. 2 we have been able to measure the electrophoretic mobilities of topoisomers

with topoisomer numbers of up to -23 (Fig. 3). These results allow a series of quantitative conclusions concerning the nature of the sequence-specific transition to be drawn. The electrophoretic mobilities of topoisomers of pDPL6 containing inserts of $d(TG)_n \cdot d(CA)_n$ correspond closely to those of the topoisomers of the vector up to a critical topoisomer number of -10 . At this point the mobility of the species declines to a mobility equivalent to that of the topoisomer having a topoisomer number -7 . We attribute this decline in mobility to the release of superhelical torsion within the vector portion of the composite plasmids. For pDHf14 (60 bp $d(TG)_n \cdot d(CA)_n$ insert) the mobility remains constant for species having topoisomer numbers -12 to -18 , and then gradually increases to a mobility slightly greater than that of the species having the topoisomer number of -10 .

For pDHf14, the helix must be unwound by a total of 11.0 ± 0.5 turns before the mobility exceeds that of the topoisomer number -7 (region A, Fig. 3). If the DNA within the inserted regions were converted to a topologically unwound state, such as a cruciform structure (as could potentially occur in the self-complementary polymer $d(GC)_n \cdot d(GC)_n$), a maximum of $60/10.4 = 5.75$ turns would be removed¹⁵. The insert $d(TG)_n \cdot d(CA)_n$ is unable to adopt a cruciform structure as the strands are not self-complementary. The additional 5.25 turns removed before the transition in the $d(TG)_n \cdot d(CA)_n$ insert is complete implies that the inserted segment adopts a left-handed helical conformation. The total winding change that would be expected if the entire 60 bp of the $d(TG)_n \cdot d(CA)_n$ insert in pDHf14 were to be converted from a right-handed 10.4 bp per turn helix to a left-handed 12 bp per turn helix is 10.7 turns ($60/10.4 + 60/12$), which agrees well with our observation. The close correspondence of the experimentally determined unwinding to the theoretically calculated number may be coincidental, as junction regions at the ends of the insertion may be unable to undergo the transition, and the helix pitch of the B

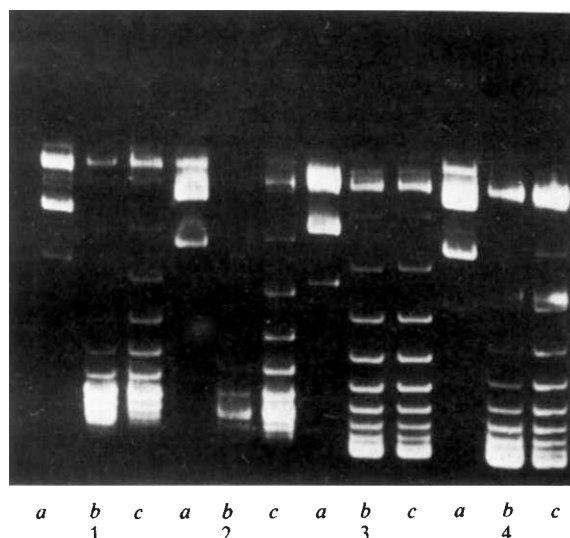


Fig. 1 One-dimensional gel electrophoresis of partially relaxed plasmid DNA. The plasmid DNA, relaxed as described below, consisted of the cloning vector pDPL6 into which a $d(TG)_n \cdot d(CA)_n$ sequence of 107 bp (lanes 1*a-c*), 60 bp (lanes 2*a-c*); or 45 bp of $d(TC)_n \cdot d(GA)_n$ (lanes 3*a-c*) had been inserted; lanes 4*a-c* contained just pDPL6 with no insert. The plasmids were relaxed in the presence of varying amounts of histones to generate the distributions of topoisomers shown in the lanes *a*, *b* and *c* (see below).

Methods: Plasmid DNA was partially relaxed as described previously¹² using 0 (*a*), 4.5 (*b*) and 6.0 (*c*) μg of core histones per 10 μg of plasmid DNA. After topoisomerase I-induced relaxation, samples were deproteinized by treatment with pronase and SDS. 1 μg of plasmid DNA was electrophoresed on a 1.5% agarose gel in TA buffer (40 mM Tris, 20 mM acetic acid, 5 mM NaAc, 1 mM EDTA) for 18 h at 3 V cm^{-1} , at 23 $^{\circ}\text{C}$ and stained with ethidium bromide ($0.5 \mu\text{g ml}^{-1}$).

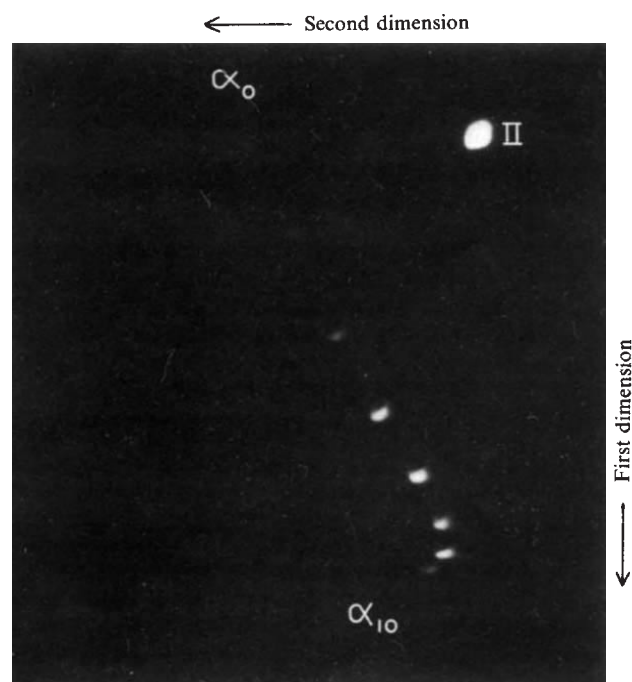


Fig. 2 Two-dimensional gel electrophoresis, the second dimension having been carried out in the presence of chloroquine. The positions of topoisomer numbers 0 (α_0) and 10 (α_{10}) are marked, as is the position of nicked plasmid DNA (II). The gel was soaked for 1 h in TA buffer containing $6 \mu\text{g ml}^{-1}$ chloroquine phosphate. The strip containing DNA was excised and placed across the top of the second dimension gel containing $6 \mu\text{g ml}^{-1}$ chloroquine in TA buffer. Electrophoresis was performed as described in Fig. 1.

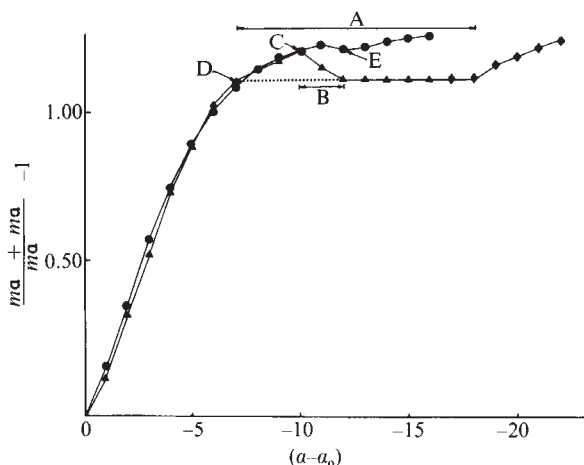


Fig. 3 Topoisomer number versus relative mobility in the first dimension. 1.5- μ g samples of partially relaxed pDHf14 and pDPL6 were electrophoresed in two dimensions as described in Fig. 2 legend. The relative mobilities of topoisomers ($\alpha - \alpha_0$) in the first dimension were measured. Topoisomer mobilities from different experiments were normalized using the distance between the most relaxed topoisomer (α_0) and topoisomer -10 as a normalization factor. Normalized mobilities ($m\alpha$) of the pDHf14 distribution. Region A, total number of negative superhelical turns unwound as a result of the Z transition. Region B, region in which the Z conformation of $d(TG)_n \cdot d(CA)_n$ may be relatively unstable. Point C, topoisomer number (-10), having the critical torsional free energy required in the molecule to initiate the transition. Point D, topoisomer number (-7) corresponding to the torsional free energy required to maintain a region of established Z-DNA. Point E, topoisomer number (-12) at which short pDPL6 sequences may adopt a Z form or where the toroidal superhelix may be converted to the interwound superhelical form. $\blacktriangle$, Averaged mobilities of pDHf14 topoisomers from three experiments. $\blacklozenge$, Mobilities of pDHf14 topoisomers from one experiment. $\bullet$, Averaged mobilities of pDPL6 topoisomers from three experiments.

and/or Z forms of $d(TG)_n \cdot d(CA)_n$ may differ from those of $d(GC)_n \cdot d(GC)_n$. These latter possibilities can, in principle, be resolved by examining the behaviour of plasmids containing a variety of lengths of the polymers.

The observation that the decline in mobility accompanying the transition occurs over the range of topoisomer number from -10 to -12, rather than being instantaneous (region B, Fig. 3) is of interest. This may indicate that a region containing less than two turns of the Z conformation of $d(TG)_n \cdot d(CA)_n$ is less stable than a longer region that has entered the Z form. We suggest that for the species having topoisomer number -11, a small region of the $d(TG)_n \cdot d(CA)_n$ insert is in a dynamic equilibrium between B and Z forms.

Using the established function relating the free energy of supercoiling to superhelix density of plasmid DNA at low ionic strength^{13,16,17} we have estimated that a critical torsional free energy of 12.1 cal per bp is required in the molecule before the transition is initiated. By making the assumption that the electrophoretic mobilities of the species reflect the torsional constraint outside the region of the insertion (in this case, in the remaining 97% of the total DNA length), we have calculated that a torsional free energy in the range 5.9–6.3 cal per bp is required to maintain an established region of $d(TG)_n \cdot d(CA)_n$ in the Z form. The amount of torsional free energy required to initiate the transition is therefore almost twice that required to maintain established regions of the Z DNA.

The reduced mobility of topoisomer number -12 (E, Fig. 3) in the control distribution may reflect a B to Z transition in small alternating purine–pyrimidine sequences that are native to pDPL6¹⁸. Alternatively, it may represent the point at which a low superhelix density form such as a toroidal form¹⁹ of the superhelix is converted to a more rigid interwound form.

A recent study of another plasmid containing $d(TG)_n \cdot d(CA)_n$ failed to demonstrate a B to Z transition in response to torsional constraint in the DNA⁹. We believe that the transition was not detected in this previous work because the topoisomer used had a superhelix density below that found here to be required to initiate the structural transition. We have found that plasmids containing various lengths of $d(TG)_n \cdot d(CA)_n$ over the range 52–120 bp require the same critical torsional free energy (~ 12.1 cal per bp) to initiate the transition, and require the same torsional free energy (~ 5.9 –6.3 cal per bp) to maintain an established region of the Z-form DNA. These values correspond to negative superhelix densities of 0.047 and 0.033–0.036, respectively, calculated using a value of 10.4 bp per turn of helix¹⁵. Recent reports from other laboratories have suggested that superhelical torsional stress alone may be sufficient to stabilize a B to Z transition in $d(GC)_n \cdot d(GC)_n$ insertions^{20–22}. Values of -0.039 and -0.03 have been suggested for the superhelical density above which the Z form predominates for this polymer. Neither of these studies considered the possibility that a higher free energy is required to initiate the transition than is required to maintain it. Accepting the latter values as those required to maintain the Z forms of $d(GC)_n \cdot d(GC)_n$, we conclude that the torsional free energies required to maintain the Z form of the two polymers are similar. We have recently found that a 20-bp insertion of $d(GC)_n \cdot d(GC)_n$ into pDPL6 undergoes a similar although not identical hysteresis in its transition between B and Z forms to that which we have observed with $d(TG)_n \cdot d(CA)_n$ insertions. However, we expect that subtle differences in structure will be found between the Z forms adopted by the two polymers.

For both $d(TG)_n \cdot d(CA)_n$ and $d(GC)_n \cdot d(GC)_n$ the superhelix densities required to stabilize the B to Z transitions are well within the range of physiological superhelix densities that have been observed in plasmids²³. The presence of a 56-bp segment of perfectly alternating $d(TG)_n \cdot d(CA)_n$ sequence ~ 300 bp downstream from the coding sequence for two mouse immunoglobulin κ -chain genes²⁴, a 12-bp segment of $d(TG)_n \cdot d(CA)_n$ on the 3' side of a mouse adult α -globin gene²⁵, a 78-bp segment located in a mouse $V_H \alpha$ immunoglobulin intron²⁶, and a 50-bp segment located within intron IV of the human cardiac muscle actin gene²⁷ provide examples of naturally occurring sequences that the present work has shown to be capable of being converted to a Z form. In view of the direct demonstration by Nordheim *et al.*⁸ that DNA in a Z conformation is found in interband regions of *Drosophila* chromosomes, it is probable that the above sequences are involved in regulatory process(es).

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Drought without water

Jean Palutikof

Drought and Man: The 1972 Case History. Vol. 1 Nature Pleads Not Guilty; Vol. 2 The Constant Catastrophe: Malnutrition, Famines and Drought.

Vol.1 by Rolando V. Garcia, Vol.2 by R.V. Garcia and Jose C. Escudero.
Pergamon: 1982. Vol.1 pp.300, £25, \$60; Vol.2 pp.204, £20, \$45.

ALTHOUGH related to events which took place over ten years ago, *Drought and Man* is of frightening relevance to the declining fortunes of the developing world. It is a study in exploitation: of poorer countries by rich ones and, within nations, of the poor classes of society by the rich. In particular, it deals with the processes by which these forms of exploitation render the poor increasingly vulnerable to the impact of drought.

Nineteen seventy-two was a year marked by the exceptionally widespread and severe occurrence of drought. In the Sahel it was the fifth consecutive year, and the water deficit was far greater than in any of the preceding years. Drought was also experienced in the USSR, Australia and India. Following a series of seminars, the International Federation of Institutes for Advanced Study and the Aspen Institute for Humanistic Studies commissioned a comprehensive study of the impact of these droughts. The findings will ultimately be presented in three volumes, of which two are currently available.

Presenting an analysis of an event a decade after it occurred, particularly one as well researched as the 1972 drought, creates certain problems. In particular, it would be all too easy to lapse into laborious repetition of existing hypotheses to explain variations in the nature and severity of the effects of drought. Garcia avoids this problem and indeed takes positive advantage of the historical perspective that the passage of time affords. He is able to take a much more global view, both geographically and thematically, than was possible for authors writing contemporary to the event. By subjecting the political and economic backgrounds of the drought to what is basically a Marxist interpretation, Garcia produces a convincing explanation of why certain societies suffered such devastating ill-effects when others escaped relatively unscathed.

Volume 1, *Nature Pleads Not Guilty*, opens with a review of the literature on the 1972 food crisis. The international grain shortages and escalating prices which began about then have commonly been blamed on the widespread occurrence of drought in that year. Garcia maintains that the situation arose not from any physical cause but because failing confidence in the dollar drove speculators into commodity trading, and because the US Government began to realign its agricultural policy.

There then follows an analysis of the causes of national differences in drought impact. By differentiating between those societies which are vulnerable to drought and those which are resilient, and tracing the historical circumstances of example nations through the colonial and post-colonial eras, Garcia demonstrates convincingly the importance of political and economic choice. The extent to which the role of climate is relegated to a secondary position is shown by the fact that the only detailed treatment of the topic is found in the last two chapters of Vol. 1. However, the essay on global climatic variability by Smagorinsky was well worth waiting for: a logical and clear exposition of a complex topic.

Volume 2, *The Constant Catastrophe*, takes as its theme malnutrition as a measure of vulnerability to drought. Dismissing official statistics as inaccurate, if not misleading, the authors investigate the real extent of malnutrition in a number of case-study examples. Countries with high endemic rates of malnutrition amongst the poorer classes are contrasted with China, where a real effort was made to spread the available resources across the whole of society.

The final chapters make some suggestions for action. At this point, however, the authors have worked themselves into something of a corner: stringent criticism of political and economic structures implies the need for radical, if not revolutionary, change. Clearly the authors did not feel in a position to make this statement, and thus the recommendations make a somewhat feeble anticlimax to what has been a fascinating story.

Potential buyers of Vol.2 should also note that it repeats, almost *verbatim*, large chunks of material from Vol.1. Only this latter volume fully merits the substantial outlay required for its purchase.

Drought and Man should be required reading for those working in the field of natural hazard impacts who approach the subject from the viewpoint of the earth sciences. Quite naturally, such a background imposes an interpretation of events which stresses, in some cases to the exclusion of all else, the role of the physical event itself. These volumes provide the economic and political context which may be lacking for those without formal training in these disciplines. Having said this, it is necessary to add that for any other audience these works provide a dangerously biased view. There is an almost total failure to recognize the importance of such physical factors as the size of the water deficit and its season-to-season persistency. In fact, the influence of climate in general is largely discounted. Haste the day when a truly balanced and interdisciplinary study of natural hazard impacts finally reaches the bookshops. □

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IMAGE
UNAVAILABLE FOR
COPYRIGHT
REASONS

Drought in Upper Volta, June 1973, the culmination of five years of abnormally low rainfall.

Photograph by L. Bouts, United Nations/UNEP

Space for the physics of plasmas

C.T. Russell

Magnetospheric Plasma Physics.

Edited by Atsuhiko Nishida.

Reidel: 1982. Pp.344. DG130, \$49.50.

SPACE plasma physics is a mature, yet dynamic, field of investigation. In such a situation preparing a book presents considerable difficulties. There are very few individuals who have a broad yet deep enough understanding of each area to cover the field by themselves, while conference proceedings are usually uneven and incomplete.

For this volume the editor has chosen an intermediate approach, selecting a small number of the key researchers to write a coordinated set of reviews. Thus each topic can be treated in the appropriate depth and the entire field can be covered. As a result, this book is clearly the best currently available account of magnetospheric plasma physics. It is, however, not without flaws. Some important aspects of the subject are completely omitted, and the style and level of each chapter is variable.

The book consists of five chapters, the two best of which are by G. Haerendel and G. Paschmann on the interaction of the solar wind with the dayside magnetosphere and by C.F. Kennel and M. Ashour-Abdalla on electrostatic waves and strong diffusion of magnetospheric electrons. These contributions comprise almost 60 per cent of the text, and in themselves justify the book's purchase. They cover their subject well and will be useful to the advanced graduate student and the specialist alike.

By contrast A.A. Galeev's chapter on magnetospheric tail dynamics, while also very good, is written on too sophisticated a level for most of whom I judge to be the potential readers of this volume. Furthermore, despite the title of the chapter, the author concentrates on the microphysical processes which are probably the consequence of the global macroscopic dynamical instability of the magnetosphere-magnetotail system.

The contribution on auroral physics by T. Sato is principally concerned with field-aligned currents and double layers, two elements of auroral physics but far from the complete story. Sato omits consideration of all wave phenomena except for a few brief remarks on auroral kilometric radiation and ion acoustic solitons. The generation of ion conics is not even mentioned. The last chapter — on the origin of magnetospheric plasma — discusses the origin of the thermal plasma of the Earth and Jupiter, i.e. the ionosphere for Earth and Io for Jupiter. However the author ignores the solar wind source almost completely and particularly the role of

convection in the outer magnetosphere in removing the upwelling thermal plasma from the ionosphere. Finally, no one seems to have been assigned the task of treating electromagnetic radiation or ULF waves; Chorus is not even mentioned in the book and the Kelvin-Helmholtz instability, kinetic Alfvén waves and ion cyclotron resonance only get passing reference.

This, then, is a good book but not a complete one. However, even the weaker chapters have good bibliographies and the inclusion of a subject index makes the book easy to use as a reference source. □

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Computer anecdotes

Simon Lavington

Breakthrough to the Computer Age.

By Harry Wulforst.

Charles Scribner's Sons: 1982. Pp.185.

\$12.95.

COMPUTER history is sufficiently recent for there to be a strong anecdotal tradition which contrasts with the analytical treatment of the serious historian. There is, however, a need for both approaches. Whilst the analyst quite rightly attempts to define trends, chart influences and pinpoint "firsts", the fact of the matter is that many early computer research groups proceeded in an *ad hoc* and relatively independent way, inventing techniques as the need arose. There was, of course, interaction and cross-fertilization but there were also personality clashes, commercial exploitation and political infighting which militated against close cooperation. *Breakthrough to the Computer Age* leans heavily towards the anecdotal and is not much the worse for that.

Harry Wulforst is a former director of public information for Sperry Univac and his book lives up to his job description. He reveals for public inspection the hitherto blurred story of the American computer pioneers J. Presper Eckert and John Mauchly, and their progression from the huge ENIAC electronic calculator, via the BINAC project, to the first UNIVAC commercial computer. It is a tale which highlights the years 1943-1951, spanning the transition from war to precarious peace and, as far as computers go, the transition from experimentation to precarious production. For the computer historian this period is still to some extent fogged by the

mists of military security and commercial sensitivity.

Mr Wulforst is at his most useful when clearing away the latter haze. He describes the financial growing pains of the infant Eckert-Mauchly computer company and its eventual takeover by Remington-Rand. There is the element of technological bluff in the delivery deadline quoted by Eckert-Mauchly for their BINAC computer, their attempts to get capital from sources as bizarre as the American Totalisator Co., and the eventual trouncing of their detractors when the first UNIVAC machine was delivered to the US Bureau of the Census in the spring of 1951. If all this can be described as a "breakthrough", then it is in the sense of a credibility breakthrough: Eckert and Mauchly became convinced of the usefulness of general purpose computers and then struggled hard to convince the paying public.

Turning to the analytical view of history, Mr Wulforst must be taken to task. He fails to appreciate the important conceptual difference between special-purpose computers such as ENIAC and general purpose stored-program computers such as BINAC and UNIVAC. To dwell on such technicalities would, however, have spoilt his breakthrough theme because he would have become involved in a serious treatment of non-American computer projects and, oh dear, a realization that the United States was not the first in the field. What little coverage he gives to other computer developments is in parts actually misleading. It is also thrown in so haphazardly that it disturbs the book's main story. But here there is a hidden parable: to Eckert and Mauchly working under great pressure in the late 1940s the activities of other computer design teams were largely irrelevant. So also with Mr Wulforst.

So let the anecdotes have the last word. There are two gems from the book which, in their different ways, have a message of encouragement for today's design teams. The first comes from the eminent mathematician John von Neumann who, in 1945, was lobbying the Navy Department in Washington for government support for building a high-speed computer at the Institute for Advanced Study, Princeton University. He said, "A group which *builds* a computer is vastly better qualified to explore its possibilities experimentally than one which obtains it readymade". The second quotation comes from Howard Aiken, a well-respected computer pioneer whose work at Harvard preceded the Eckert-Mauchly enterprises. In 1950 Aiken was of the opinion that "no more than six computers would ever be sold in the commercial market". □

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American Antarctica

Antarctic Wildlife, with photographs by Eric Hosking and text by Bryan Sage, has just become available in the United States. Publisher is Facts on File, price \$22.95. For review see *Nature* 300, 556 (1982).

Teaching mechanisms

Joseph G. Gall

Molecular Biology of the Cell.

By Bruce Alberts *et al.*

Garland: 1983. Pp. 1,146. Hbk \$33.95;
pbk £12.50.

THE nineteenth-century promise that the study of cells, their components and their products would illuminate all aspects of biology has been amply fulfilled in recent years, so much so that writing a comprehensive treatise on cell biology is a formidable task, some would say impossible. It is difficult enough to bring together the body of biochemical information on cytoplasmic organelles or to concentrate on the molecular biology of the gene. But how is one to summarize all of that information and then show how it relates to development, neurobiology, immunology and a half-dozen other major fields?

The authors of *Molecular Biology of the Cell* have done it as a committee of six — Bruce Alberts, Dennis Bray, Julian Lewis, Martin Raff, Keith Roberts and James Watson. What they have given us is 1,146 pages of detailed information and interpretation covering virtually all those aspects of biology currently amenable to molecular and cellular analysis. And they have done it remarkably well.

Nineteen chapters are divided into three major headings: "An Introduction to the Cell" (four chapters), "The Molecular Organization of Cells" (nine chapters) and a final section entitled "From Cells to Multicellular Organisms" (six chapters). Each chapter is divided into a small number of major headings, which in turn are discussed under subheadings. The subheadings consist of simple statements or questions in the style so popular in recent

years for the titles of journal articles (e.g. "The DNA replication fork is asymmetrical", "Carbohydrate structures are modified in the Golgi apparatus", "The genetic control of development is best studied in *Drosophila*"). Each subheading is then discussed in several paragraphs, with numerous references to recent, original literature and with diagrams and photographs. The illustrations have been carefully selected for their pictorial and aesthetic quality as well as for content. Nearly every major point in the text is accompanied by a diagram or photograph on the same page, enabling the reader to move effortlessly from text to picture and back.

The format of numerous small sections is well suited for simple statements of fact or for well-established generalizations, and as a didactic mechanism it focuses attention on a single issue at a time. It is somewhat less appropriate for major questions or where an important issue is still in dispute. Judiciously used, this format leaves the overall impression that problems are resolvable, at least in principle. The sense that molecular biology has clarified old issues and will continue to do so is a major theme of the work.

The amount of general information brought together here is impressive. Much of what is traditionally found in textbooks of histology and developmental biology is mentioned, and there are long chapters on the immune system, the nervous system and the special features of plant cells. Such an ambitious undertaking could easily have gone wrong. It could have given us an encyclopaedia useful for reference but not for general reading, or a disjointed compendium of good, bad and indifferent chapters. Someone has seen to it that neither of these fates befell this work. There is remarkably little duplication, the style is uniform throughout and the relative weight given to different sections has been

thought out carefully. The authors and editors have also done a good job in eliminating minor errors. Much credit must go to the extensive reviews and contributions of some 75 additional people listed in the acknowledgements. In the end, however, this book is a tribute to the ability and effort of the six main authors.

For whom is the book intended? The authors say it is principally for students taking a first course in cell biology, be they undergraduates, graduate students or medical students. Certainly the level of treatment is suitable for these groups, but the amount of material would be overwhelming for a typical first course. As a practising cell biologist I found much that was new to me except in those few areas I deal with regularly. My suggestion is that this text would be ideally suited for a year-long, middle-level course in which the traditional units in cell biology, developmental biology and molecular genetics are restructured into a single integrated presentation. There is much duplication of effort in these courses as currently taught and this text will serve a useful function if it spurs efforts at consolidation.

Molecular Biology of the Cell shows what can be done when six experts pool their talents under strong editorial leadership — an integrated, authoritative account presented in a thoroughly readable format and full of informative and attractive figures. It will be a long time before you find a better buy. □

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Wanted: a new view of mountains

Janet V. Watson

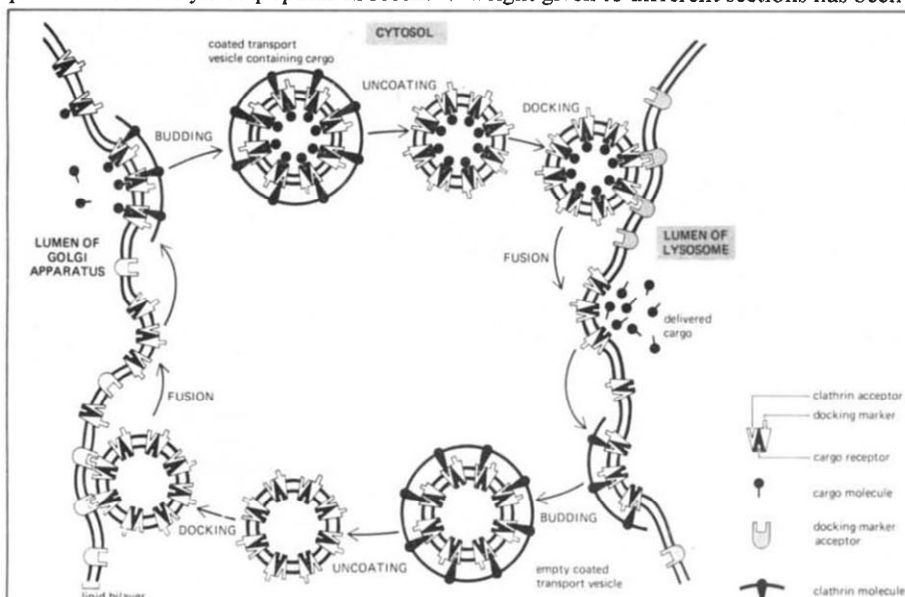
Orogeny.

By Akiho Miyashiro, Keiiti Aki and
A.M. Cclâl Şengör.

Wiley: 1982. Pp. 242. Hbk £19, \$35.95;
pbk £8.40, \$17.95.

LIKE other sciences that established themselves in the eighteenth and early nineteenth centuries, geology has its roots in the intellectual life of Europe. Unlike other sciences, its development was determined to some extent by the structure of the European continent itself. It is fascinating to speculate on how the subject would have evolved if the founding fathers had gained their knowledge mainly from observations in Africa or Australia.

The unspoken assumption that Europe is the norm has had a particular influence on the development of ideas about orogeny, since it gave undue prominence to the atypical Alpine orogenic belt. This review of "theories of orogeny" by two



Graphic design in pedagogy, from *Molecular Biology of the Cell* — a highly schematic diagram of a possible mechanism allowing the targeting of coated transport vesicles to a specific intracellular membrane. The original illustration is in two colours.

Japanese and one Turkish author might therefore be expected to offer a new perspective. The book, however, is a translation of one published a few years ago in Japanese and therefore seems more concerned to retell the evolution of ideas of European-American origin than to redress the balance. In these circumstances, it is not surprising that Professor Miyashiro's treatment (in Chapter 3) of themes which are drawn principally from studies of the circum-Pacific orogenic belt (notably the concept of paired metamorphic belts, to which he contributed) has a freshness rather lacking elsewhere.

In general, the stated objective — to provide an introduction to theories of orogeny — seems at once too restrictive and too vague to give the book a satisfactory shape. I find it hard to imagine what kind of reader the authors have in mind. In the opening

chapter ("Classical Theories") Şengör homes in on the great masters of Alpine geology, placing tectonics firmly at the centre of the stage. Next, "Theory of Orogeny based on Plate Tectonics" provides a standard exposition of the plate tectonic hypothesis that might fit into any general textbook. The fourth chapter, "Mechanisms of Orogeny", abandons consideration of the development of ideas in favour of a quite advanced treatment of the mechanical properties of plates and the stresses acting on them. The final contribution, on Precambrian orogenies, is little more than perfunctory.

The view from Japan, which might have proved so illuminating, never quite materializes. □

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The succession of ecologists in North America

William Coleman

Saving the Prairies: The Life Cycle of the Founding School of American Plant Ecology, 1895–1955.

By Ronald C. Tobey.

University of California Press: 1982.

Pp.315. \$32.50, £21.25.

THE PRAIRIES referred to in the title of Ronald Tobey's book were the grasslands of western North America, the saviours the offspring of that peculiar breed of social philosopher, nature enthusiast and aspiring scientist characteristic of the late nineteenth-century United States. Convinced that the prairies could withstand virtually any insult, the founders of American plant ecology discovered to their distress that the great restorative, plant succession leading to a return of the regional climax, could be and in fact had been dramatically disturbed. The double blow of rapid settlement accompanied by wanton exploitation and the Great Drought destroyed a plant-community that had prospered on the middle and high plains for many thousands of years. Lost plant communities were followed by a loss of scientific conviction, hard upon the heels of which was the disruption of the Second World War. After 1945 came the re-creation of plant ecology along new lines, notably based upon the notion of the ecosystem — most cogently expressed by Arthur G. Tansley in England — and upon the emerging principles of productivity ecology.

These several events constitute a somewhat complicated story which Ronald C. Tobey relates in a by no means uncomplicated

manner. Several potentially substantial books lurk within this volume and the general sense of busyness is not reduced by the author's keen insistence on what he seems to regard (p.8) as his major innovation, namely, systematic application of citation analysis as a tool for the identification and description of a school of scientific thought. There is a disturbing sense of the obvious, however, in the principal conclusion which Tobey draws from this numerical material — an "enduring determinant of citation behaviour is the intellectual interest of the scientists in the literature" (p.149).

The "School" in question was that of Nebraska botanists founded by Charles E. Bessey during the 1890s and soon raised to larger dimensions by a truly exotic bloom, Frederic E. Clements. Clements viewed the botanical formation as the primary unit of ecological analysis; he, his associates and students developed the quadrat technique for studying the species constitution of a formation as a means by which to introduce numerical rigour into the seemingly subjective domain of floristic description. And he adapted the ancient metaphor of the social organism to fit the grassland and other formations, and thus by 1916 had come to insist upon the unidirectional trend of plant succession. If succession under given climatic conditions, such as those encountered in central North America, invariably returns a community to its climax, we need not fear that even serious human disturbance of the region can diminish its recuperative powers.

Unfortunately, the facts suggested another conclusion and it was Clements's foremost student, John Weaver, who made it widely known. The settlers had indeed destroyed the native plant community and the only hope for a region which, with its plant cover gone was also rapidly losing its soil, was for man himself to attempt to restore the original conditions or at least to retard the process of deterioration. Weaver well understood this point and Clements,

too, embraced it with enthusiasm even though it shattered the cognitive heart of his programme; the Nebraska school prospered during the 1930s by providing expertise and training scientists for government-sponsored programmes for prairie rescue, a salvation that now was seen not to reside in the nature of things but to depend upon human intervention. There is a fine moral here for those who rush to apply the latest and seemingly most sound scientific conclusions. The Clements school at different periods entertained conflicting environmental approaches and did so always with the conviction of the purity of its scientific principles. The prairies, however, were unforgiving and the optimistic doctrine of grassland succession and inevitable recovery collapsed only as the prairies themselves were exhausted.

Tobey provides a comprehensive account of the influential Nebraska school from its birth to its demise. His discussion of Tansley, a decisive figure in British ecology and a shrewd critic whose social and political views were as significant for his scientific work as were those of Clements for his outlook on ecology, is outstanding. Nebraska's early rival, the Chicago school of ecology led by Henry Chandler Cowles, working out the programme of Eugenius Warming, receives notice but, unfortunately, little close scrutiny. The whole domain of autecology is not discussed. These limitations may indeed reflect historical reality: perhaps the Clementsonian successionist programme did serve to establish the "founding school of American plant ecology". But rivals also existed and the reasons why their views did not prosper remain unclear. Certainly Tobey's claim that the triumph of the Nebraskans is to be explained "sociologically" (that is, by the successful creation of a large-scale research and training programme, a distinctive feature of the aggressive new American land-grant universities), is part of the answer. And so, too, is the optimism that informed the early fancies of Frederic Clements regarding the wonderful ever-vivifying garden in the American west. These, however, are still but incomplete answers and they necessarily return and confine attention to Nebraska.

Within that framework, Tobey has prepared a valuable introduction to the general theme of the formation and transformation of disciplines in the natural sciences, and to the interrelated issues of ecological understanding and environmentalist action (really, for long years, non-action). *Saving the Prairies* should be widely read and, more importantly, its suggestions extended and silences explored. □

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